Adiponectin: a novel circulating anti-thrombotic factor in humans?

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Abstract

Adiponectin is an adipocyte-derived pro-inflammatory adipokine that regulates metabolic homeostasis, although it has been reported to modulate platelet function and thrombosis. In this study the effects of globular adiponectin (gAd), a novel platelet agonist that stimulates platelet aggregation was characterised. While it had been previously noted that gAd stimulated platelet aggregation through a tyrosine kinase-dependent mechanism, in this thesis we further elaborated the mechanisms underlying gAd-induced platelet aggregation by investigating the role of platelet secondary mediators, granule secretion and intracellular signalling events using a series of receptor antagonists and inhibitors.

The experimental data demonstrated that gAd-stimulated aggregation required adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2) to achieve the maximal response. Consistent with this, threshold concentrations of gAd could synergise with ADP. However, using receptor antagonists this synergy was found to occur via the G_i-coupled $P2Y_{12}$ receptor and not G_q-coupled $P2Y_1$. Since the secretion of ADP from platelet dense-granules is driven by protein kinase C (PKC), we examined the role of PKC in gAd-mediated platelet aggregation. Ablation of PKC using a general inhibitor reduced platelet aggregation and abolished dense-granule secretion stimulated by gAd. Importantly, the aggregation response and dense-granule secretion under conditions of PKC inhibition could be partially restored by ADP acting through the $P2Y_{12}$ coupled G_i receptor and by adrenaline acting through the G_z-linked α_{2A}-adrenoceptor. Further examination of this pathway revealed that the activation of phosphatidyl-inositol-3-kinase (PI3K) was required to restore secretion. To clarify the role of PI3K in this process we measured Akt phosphorylation as a marker of PI3K activity. We found that ADP and adrenaline potentiated gAd-induced Akt phosphorylation which could be inhibited with a selective PI3Kβ isoform inhibitor and to a lesser degree α isoform inhibitor. These data demonstrate that gAd requires the release of platelet secondary mediators to induce full platelet aggregation and the potentiation of initial

aggregation occurs through G-protein coupled receptors linked to G_i-coupled G-proteins. Furthermore, the stimulation of G_i/G_z-coupled G-proteins can potentiate platelet aggregation by stimulating secretion through a PKC-independent manner.

Since adiponectin accumulates at sites of vascular lesions, the ability of immobilised gAd to support platelet adhesion was examined. GAd supported the adhesion of platelets under static and flow conditions. Adhesion under static conditions was inhibited by the glycoprotein VI (GPVI) blocking antibody, 10B12, but not ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N',-tetraacetic acid (EGTA), suggesting a GPVI-dependent and integrin-independent mechanism.

Together these data significantly improve our understanding of the mechanisms by which gAd may promote unwanted platelet aggregation and adhesion at sites of vascular injury.

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In the loving memory of my late father Naheed Ahmed Yousaf and grandfather Chaudhary Mohammed Yusaf

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Declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources.

Chapter 1: General Introduction

Platelets are specialised blood cells that play a central role in supporting haemostasis. However, improper platelet activation can lead to thrombosis and the development of pathological diseases such as acute coronary syndrome that includes myocardial infarction (Ruggeri, 2002, Davi and Patrono, 2007). Platelets circulate in the blood in a quiescent state and can undergo rapid activation at sites of vascular injury to form a platelet plug. Platelet activation involves a complex association between exposed components of the extracellular matrix proteins and platelet surface receptors. This association is dependent on the complex interplay of adhesion and signalling molecules that regulate platelet function. The critical role of platelets in both health and disease has led to an intensive search for new factors that may influence platelet function. In this thesis, one such protein called adiponectin was investigated as a novel regulator of platelet activity. Adiponectin is an adipokine produced exclusively by adipose tissue (Kadowaki et al., 2006). The biological function of adiponectin is complex and varied. Its primary function is as an insulin-sensitising hormone and therefore plays a role in carbohydrate and lipid metabolism, although anti-diabetic, anti-inflammatory and vascular protective actions have also been described (Okamoto et al., 2002, Ouchi and Walsh, 2007, Zhu et al., 2008). This chapter will review the literature regarding platelet function, in particular focusing on molecular pathways involved in platelet activation and any possible platelet-adiponectin interaction will be explored.

1.1 Blood platelets

1.1.1 Platelet formation

Platelets are small non-nucleated cells that were first described by Osler in 1874 (Osler, 1874). At that time some individuals prescribed to the view that platelets could be parasitic organisms found in the blood. Almost ten years after platelets were first described, Bizzozero (1882) correctly characterised platelets anatomically and their role in haemostasis and thrombosis (Bizzozero, 1882). Wright (1906) discovered that platelets were produced from progenitor cells called megakaryocytes (Wright, 1906). Like other blood cells, megakaryocytes are derived from hematopoietic stem cells in the bone marrow. Mature megakaryocytes rapidly fill with platelet-specific proteins, organelles and membrane systems that will ultimately be subdivided and packaged into platelets (Kaushansky, 1995). Small fragments termed pro-platelets containing these platelet proteins and organelles are released into circulation through mechanisms requiring thrombopoieitin (Kaushansky et al., 1994). Each of these pro-platelet processes can give rise to 2000-5000 new platelets, which helps to maintain a normal human blood platelet concentration of $150x10^9$ to $450x10^9$ platelets/L. Platelets have a relatively short half-life of 8 to 10 days in the blood and are removed from the circulation by the spleen (Weiss, 1975).

1.1.2 Platelet structure

Much of platelet structure has been defined by electron microscopy. Human platelets are characterised by their disc like shape and are approximately 2-5 µm in diameter (White, 1979) [Figure 1.1]. Platelets are surrounded by a plasma membrane which is composed of phospholipid bilayer, proteins and sugars (White, 1979). The membrane forms multiple invaginations into the cytoplasm to form the open canalicular system (OCS), which facilitates platelet spreading and granule release (White, 1979, White and Clawson, 1980). Platelets in addition to the OCS have separate membrane network called the dense tubular system (DTS). These are believed to be remnants of smooth endoplasmic reticulum from the parent megakaryocyte that enables the storage of calcium (Gerrard et al., 1978). The release of calcium ions (Ca^{2+}) from the DTS is proposed to drive platelet activation pathways (Varga-Szabo et al., 2009). An enzyme involved in TxA_2 synthesis, cyclo-oxygenase-1 (COX-1) is also found in the DTS (Gerrard et al., 1978).

1.1.3 Platelet organelles

In addition to the tubular systems, platelets contain three main types of storage granules; alpha-granules, dense-granules and lysosomes (Holmsen and Day, 1968, White, 1979, Harrison and Cramer, 1993). There are approximately 40-80 alpha-granules found per platelet with a diameter of 0.4 µm (Harrison and Cramer, 1993). In contrast, 4-5 dense-granules are found per platelet and are much smaller than alpha-granules, with a diameter of 0.10-0.15 µm (White, 1968). Alpha-granules mainly contain adhesive proteins which include von Willebrand factor (vWF), P-selectin and fibrinogen [Table 1.1] and are released during platelet activation. The release of these contents has multiple effects on the haemostatic process by enhancing the coagulation response (fibrinogen, Factor VII), endothelial repair (PDGF) and the inflammatory response (P-selectin) (Abrams, 2008). The contents of dense-granules are also released during platelet activation, which includes ADP, adenosine triphosphate (ATP), serotonin and Ca^{2+}. These agents are all thought to enhance the platelet aggregation response (Charo et al., 1977). Platelet secretion and shape change are both energy-dependent processes, and consequently platelets contain mitochondria and glycogen granules to provide required ATP (Weiss, 1975).

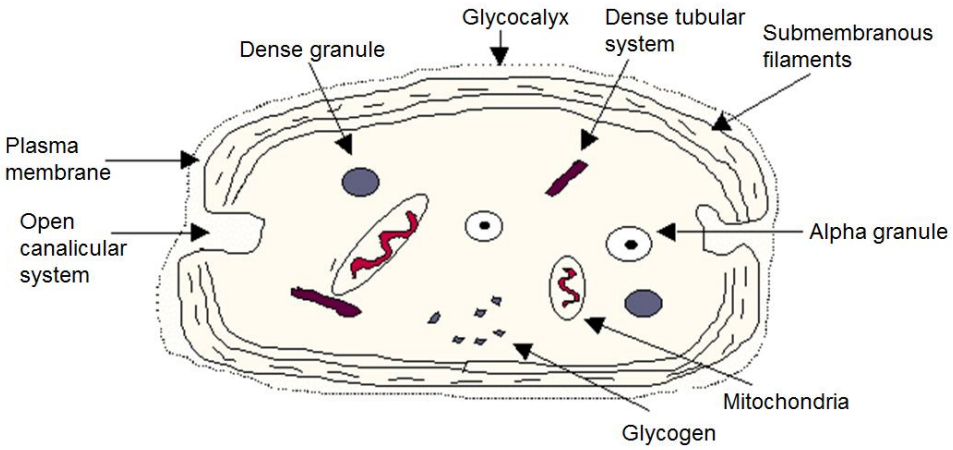


Figure 1.1: Ultrastructure of platelet. The platelet is composed of an outer plasma membrane which forms invaginations to form the open canalicular system. The platelet also contains alpha and dense-granules whose contents are released upon platelet activation. Adapted from www.platelet.net/platelets/platelets.html (Platelets.net, 2011).

Alpha granules	Dense granules
vWF	ADP
Factor V	ATP
Fibrinogen	Serotonin
P-selectin	Ca^{2+}
Thrombospondin	
PDGF	
Platelet factor IV	

Table 1.1: Summary of important platelet granule contents.

1.1.4 Platelet receptors

Platelets have a range of important plasma membrane receptors that recognise various activating factors and are responsible for platelet activation. Platelet surface receptors can be divided into several groups; integrins, G-protein coupled receptors, tyrosine kinase-linked receptors and leucine rich-repeat family receptors. Important receptors that are involved in platelet activation include the collagen receptors GPVI and the integrin $\alpha_2\beta_1$, vWF receptor complex GPIb-V-IX and the fibrinogen receptor $\alpha_{IIb}\beta_3$ (Clemetson, 2003, Gibbins, 2004, Watson et al., 2005). A summary of important platelet surface receptors is shown in Table 1.2.

Many platelet surface receptors are coupled to heterotrimeric G-proteins that are involved in platelet activation and inhibition. These include the ADP receptors, $P2Y_1$ and $P2Y_{12}$ and thrombin receptors, protease activated receptors (PAR) 1 and 4 (Offermanns, 2006). G-proteins consist of an α-subunit and an undissociable β and γ-subunit complex (Gilman, 1987, Kleuss et al., 1993). The α-subunit contains a guanine nucleotide binding site that is occupied by guanosine diphosphate (GDP) and is exchanged by guanosine triphosphate (GTP) once receptor activation has taken place (Gilman, 1987). Binding of GTP to the α-subunit induces a conformational change resulting in the dissociation of the α-subunit and the βγ-subunit complex. The dissociated α-subunit and the βγ complex interact with downstream effectors such as phospholipase C (PLC). The α-subunit contains intrinsic GTPase activity which hydrolyses GTP back to GDP and results in the re-dissociation of the GDP bound α-subunit to the βγ complex, thereby restoring the G-protein back to its resting state (Sprang, 1997). G-proteins are identified on their α-subunits, $G\alpha_s$, $G\alpha_q$, $G\alpha_i$, $G\alpha_{12}$ and $G\alpha_{13}$ are all present in human platelets (Simon et al., 1991). Studies on gene deletions in mice have shown $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12}$ family members are involved in platelet activation (Offermanns, 2000, Offermanns, 2006). Platelets also contain $G\alpha_z$, a G-protein coupled to α_{2A} receptor of adrenaline involved in the inhibition of adenylyl cyclase (Hayes et al., 1999). $G\alpha_s$-proteins are coupled to prostacyclin receptor, IP and act to

increase cyclic adenosine monophosphate (cAMP) formation via adenylyl cyclase activation and promote platelet inhibition (Gorman et al., 1977, Tang and Gilman, 1991, Yang et al., 2002). G-protein receptors coupled to $G\alpha_i$ subtype, mainly $G\alpha_{i2}$ and $G\alpha_{i3}$ have an inhibitory effect on adenylyl cyclase (Murayama, 1996).

Agonist	Receptor	Receptor type	Signalling pathway	Copy number
ADP	$P2Y_1$	GPCR	G_q/G_{12}	150
	$P2Y_{12}$	GPCR	G_i	600
Thromboxane	$TP\alpha/\beta$	GPCR	$G_q/G_{12}/G_{13}$	1000
Adrenaline	α_{2A}-receptor	GPCR	G_z	300
Thrombin	PAR1	GPCR	$G_q/G_{12}/G_{13}$	2500
	PAR4	GPCR	$G_q/G_{12}/G_{13}$	2000-3000
Prostacyclin	IP	GPCR	G_s	-
Collagen	GPVI	Ig	Tyrosine kinase	5000*
	$\alpha_2\beta_1$	Integrin	Tyrosine kinase	2000-4000
vWF	GPIb-V-IX	Leucine-rich	Tyrosine kinase	50,000
Fibrinogen	$\alpha_{IIb}\beta_3$	Integrin	Tyrosine kinase	60,000-80,000

Table 1.2: Summary of important platelet surface receptors. Platelets express surface receptors which are involved in platelet inhibition and activation pathways. *(Best et al., 2003)

1.2 Platelet function

Platelets circulate in the blood in an inactive state and can undergo rapid activation at sites of vascular injury. Platelets are normally separated from the underlying thrombogenic extracellular matrix proteins by endothelial cells. This lining of endothelial cells provides a non-thrombogenic surface, which helps to preserve platelets in their quiescent state. Platelets are forced towards the endothelium due to the haemodynamic forces exerted upon them (Malone and Morris, 1978, Zhao et al., 2007). This process of margination allows platelets to be in the perfect position to detect any damage or changes in the lining of endothelial cells. Upon injury, the endothelial barrier is damaged and extracellular matrix proteins such as vWF and collagen are exposed. Platelets have a range of important plasma membrane receptors that recognise these activating factors and are responsible for platelet activation (section 1.1.4). Platelet activation has been divided into three stages; initiation, extension and perpetuation [Figure 1.2]. These stages are further broken down into platelet adhesion, secretion and aggregation.

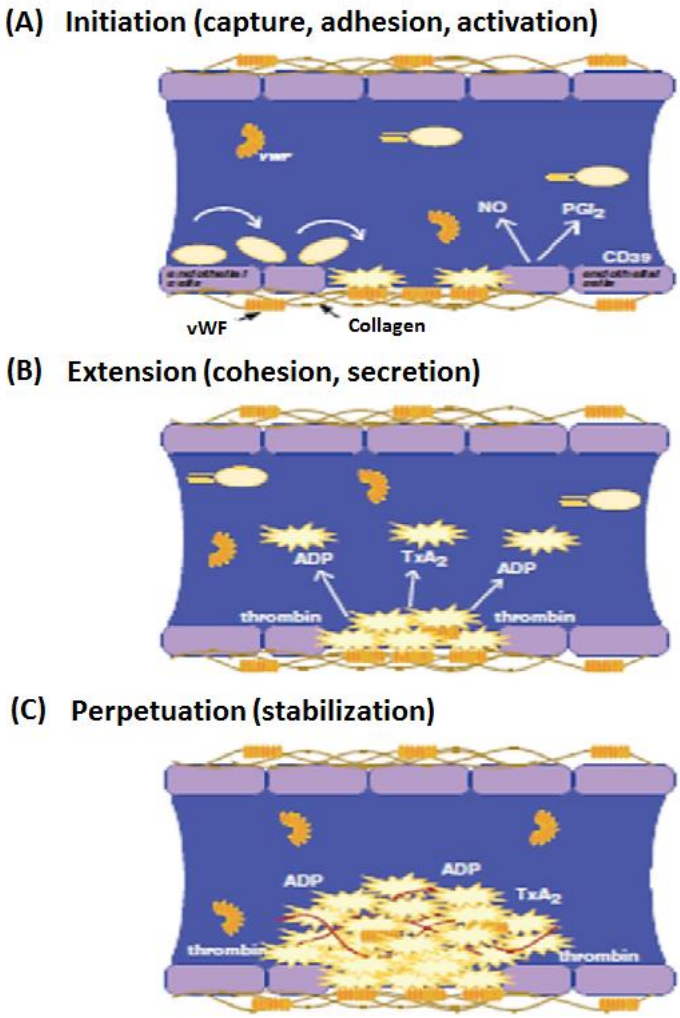


Figure 1.2: Stages of platelet plug formation. (**A**) The platelet plug formation is initiated by the exposure of vWF and collagen at the site of injury which allows circulating platelets to adhere and spread and form a platelet monolayer. (**B**) The local secretion of ADP and TxA_2 by the adhered platelet monolayer in conjunction with thrombin generation reinforces platelet activation allowing the recruitment of additional platelets. (**C**) The final stage of platelet plug formation is associated with the stabilisation and growth of the platelet plug. Figure adapted from Platelets, Second Edition, 2007 (Michelson, 2007).

1.2.1 Platelet adhesion

The initial step in the haemostatic process is platelet adhesion with exposed elements of extracellular matrix proteins such as collagen or vWF at the site of vascular injury. At very high shear rates found in small arteries and arterioles, platelet adhesion requires the interaction of vWF with platelet GPIb-IX-V receptor complex (Ikeda et al., 1991). vWF is a large multimeric plasma protein primarily found in the endothelium, sub-endothelial tissue and platelet alpha-granules (Sadler, 1998). vWF is able to interact with a number of proteins including coagulation factor VIII and collagen (Ruggeri and Zimmerman, 1981, Rand et al., 1991). Factor VIII is found in circulation bound to vWF and is rapidly degraded when not bound (Morton et al., 1983). Plasma vWF is able to bind to exposed collagen fibres through a conformation change with the interaction of vWF A3 domain with collagen (Lankhof et al., 1996, Tsai, 1996).

This interaction between vWF and GPIb-IX-V receptor complex is reversible and insufficient for stable adhesion (Ikeda et al., 1991). It is thought that the high shear stress causes vWF, GPIb-V-IX or both to change their conformation to allow the platelet to adhere. This reversible process is called tethering and slows the platelet down sufficiently to allow stable adhesion to take place. Firm platelet adhesion requires the interaction of platelet integrins and collagen [Figure 1.3]**.** Integrins are a group of ubiquitously expressed heterodimeric receptors that play a key role in cell-cell contact, cell-ligand contact and cell spreading (Shattil and Newman, 2004). Under normal circumstances integrins are found in a low affinity state unable to bind their ligands. However, upon cell activation a conformational change ensues that allows binding to extracellular proteins. In platelets, integrins $\alpha_2\beta_1$ or $\alpha_{IIb}\beta_3$ play major roles, although there is evidence that integrins $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_v\beta_3$ can also participate (Varga-Szabo et al., 2008). Collagen can bind directly to $\alpha_2\beta_1$ or indirectly to $\alpha_{IIb}\beta_3$ via vWF to facilitate stable adhesion. In both cases inside-out signalling events from other receptors are required to convert these integrins from a low affinity state to a high affinity state (Clemetson and Clemetson, 2001).

For firm platelet adhesion to occur under high shear requires intracellular signals from GPVI and outside-in signalling resulting in the transformation of the platelet from a discoid to a more spherical shape with the extrusion of filipodia (Inoue et al., 2003, Nieswandt and Watson, 2003). At low shear rates, collagen itself can support platelet adhesion in the absence of vWF through $\alpha_2\beta_1$ (Saelman et al., 1994). Thus, regardless of the shear rate the platelet-collagen interaction is critical to the haemostatic response.

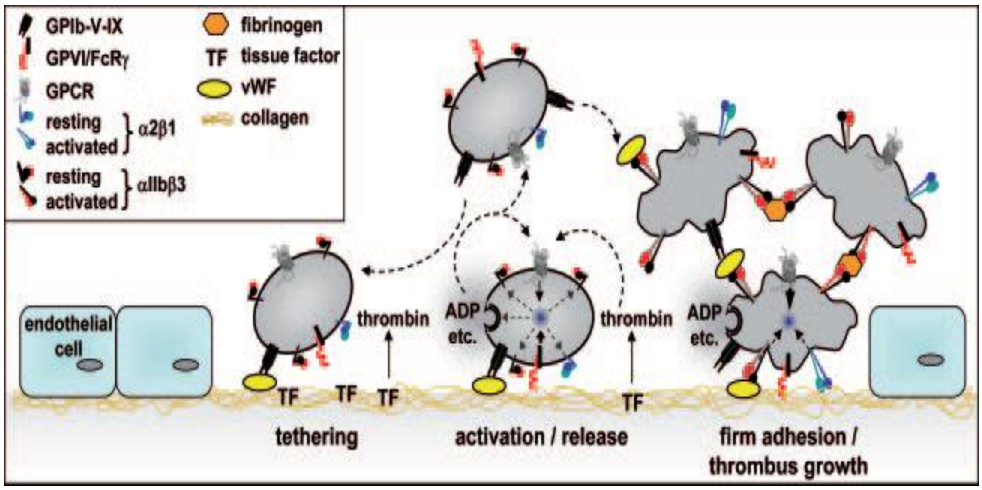


Figure 1.3: Proposed model of platelet adhesion in high shear. Circulating platelets are slowed down by the association of vWF to GPIb-V-IX receptor complex which enables the platelet to roll along the surface. Firm platelet adhesion is mediated through the interaction of collagen between GPVI and integrin $\alpha_2\beta_1$. Integrin activation in combination with the release of soluble platelet agonists such as ADP and TxA_2 reinforce platelet activation by outside-in signalling resulting in the transformation of the platelet from a discoid shape to spherical with the extrusion of filipodia (Varga-Szabo et al., 2008).

1.2.2 Platelet-collagen interaction

Platelets are activated at the site of vascular injury when exposed to activating factors, such as collagen. There are at least 9 types of collagen found in the vascular system; namely types I, III, IV, V. VI, VII, XII, XIII and XIV with collagen I, III and IV forming a major component of the blood vessels (Barnes and Farndale, 1999, Nieswandt and Watson, 2003). Typically, collagen is composed of a triple helix structure that contains a repeat motif glycine-proline-X (GPX), where X can be any amino acid but frequently hydroxyproline (O) (Knight et al., 1999, Smethurst et al., 2007). The GPO repeat motif is found abundantly in collagen and makes up approximately 10% of collagen type I and III (Farndale et al., 2004). Furthermore, synthetic peptides have been developed such as cross linked-collagen related peptide (CRP-XL) which are composed of the GPO motifs and shown to mimic collagen-stimulated platelet activation (Knight et al., 1999). Platelets express surface receptors that can bind to collagen such as GPVI and integrin $\alpha_2\beta_1$. Platelets can also bind indirectly to collagen via its interaction with vWF to GPIb and $\alpha_{IIb}\beta_3$ (Nieswandt and Watson, 2003).

1.2.2.1 Structure of glycoprotein VI

GPVI is a 58 kDa platelet membrane immunoglobulin (Ig) family receptor that has been described as the central receptor in platelet-collagen interactions (Nieswandt and Watson, 2003). GPVI is composed of two Ig domains, a mucin rich stalk and a cytosolic tail that is 51 amino acids long [Figure 1.4] (Clemetson and Clemetson, 2001). The cytosolic tail of GPVI contains motifs that can bind to calmodulin and Src-kinases (Watson et al., 2005). GPVI is constitutively associated to a disulfide-linked Fc receptor (FcR) γ-chain homodimer in the membrane via a salt bridge between charged arginine amino acid within the transmembrane and cytosolic tail (Tsuji et al., 1997, Ezumi et al., 2002). Importantly, this association is required for GPVI surface expression (Nieswandt et al., 2000). Each copy of the FcR-γ homodimer contains an immunoreceptor tyrosine activation motif (ITAM) which provides a docking site (YxxL) for Src homology (SH) 3 containing Src tyrosine kinase family such as Lyn and Fyn and is essential for GPVI activation (Suzuki-Inoue et al., 2002).

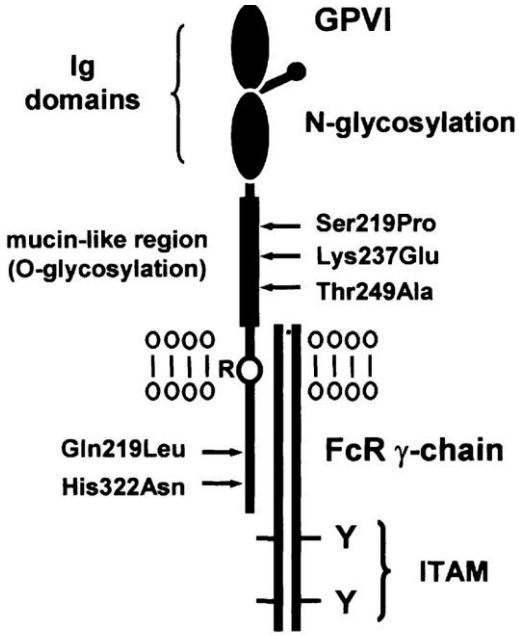


Figure 1.4: Structure of collagen receptor glycoprotein VI. GPVI is composed of two Ig domains, a mucin rich stalk and a cytosolic tail. GPVI is coupled to a disulfide linked FcRγ-chain homodimer in the membrane via a salt bridge between charged arginine amino acid within the transmembrane and cytosolic tail (Nieswandt and Watson, 2003).

1.2.2.2 Platelet activation through ligation of glycoprotein VI

The present model for GPVI activation is based on the ability of distinct transmembrane regions of GPVI and the attached FcRγ-chain complex to associate with other proteins that enable inside-out signalling which support the activation of $\alpha_{IIb}\beta_3$ from a low to high affinity state (Kamiguti et al., 2000). Binding of collagen to GPVI results in the dimerisation of GPVI receptors with its associated FcRγ-chain [Figure 1.5] (Tsuji et al., 1997, Arthur et al., 2007). The GPVI cytosolic tail contains recognised sequence motifs for binding with SH3 domain Fyn and Lyn (Ezumi et al., 1998, Bori-Sanz et al., 2003). These Src-family tyrosine kinases phosphorylate tyrosine residues in the associated FcRγ-chain ITAM that allow the binding and activation of tyrosine kinase, spleen tyrosine kinase (Syk) (Gibbins et al., 1997, Quek et al., 2000). Recently, the protein tyrosine phosphatase CD148 has shown to a play a role in regulating Fyn and Lyn downstream of GPVI by dephosphorylating an inhibitory tyrosine residue and returning the protein back into an inactive conformation (Senis et al., 2009).

Syk contains a SH2 domain which can recognise the phosphorylated tyrosine residues within the ITAM (Yanaga et al., 1995). Mouse platelets which are deficient in Syk are found to be unresponsive to collagen and therefore confirming the critical role of Syk in collagen-stimulated platelet activation (Poole et al., 1997). GPVI-mediated activation of Syk has been shown to mediate phosphorylation of a number of downstream adaptor proteins such as linker for activation of T-cells (LAT) (Gibbins et al., 1996, Asazuma et al., 2000). The activation of LAT is an important stage in platelet GPVI activation that allows the recruitment of SH2 domain containing proteins and formation of a signalosome [Figure 1.5] (Asazuma et al., 2000). The recruitment of Grb2 (growth factor receptor bound protein 2), Gads (Grb2 related protein downstream of Shc) and SLP76 (SH2 domain containing leukocyte proteins of 76 kDa) are critical for the activation of PLCγ2 isoform (Gross et al., 1999, Liu et al., 1999, Yablonski et al., 2001). LAT also binds to PI3K and brings PLCγ2 in closer proximity to PI3K derived phosphoinositide products which bind to the pleckstrin

homology (PH) domain of PLCγ2 and therefore support its localisation to the membrane [Figure 1.5] (Gibbins et al., 1998). Activated PLCγ2 results in the formation of secondary messengers, inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts as a ligand to open ligand gated Ca^{2+} channels in the platelet plasma membrane and Ca^{2+} channels in the platelet DTS (Kaibuchi et al., 1983). This increase in cytosolic Ca^{2+} concentration in combination with DAG leads to the activation of PKC isoforms that drive ADP and TxA_2 secretion (Tyers et al., 1988, Quinton et al., 2002). The release of ADP and TxA_2 bind to their own receptors and generate signals that support the activation of the platelet (Nieswandt and Watson, 2003, Watson et al., 2005).

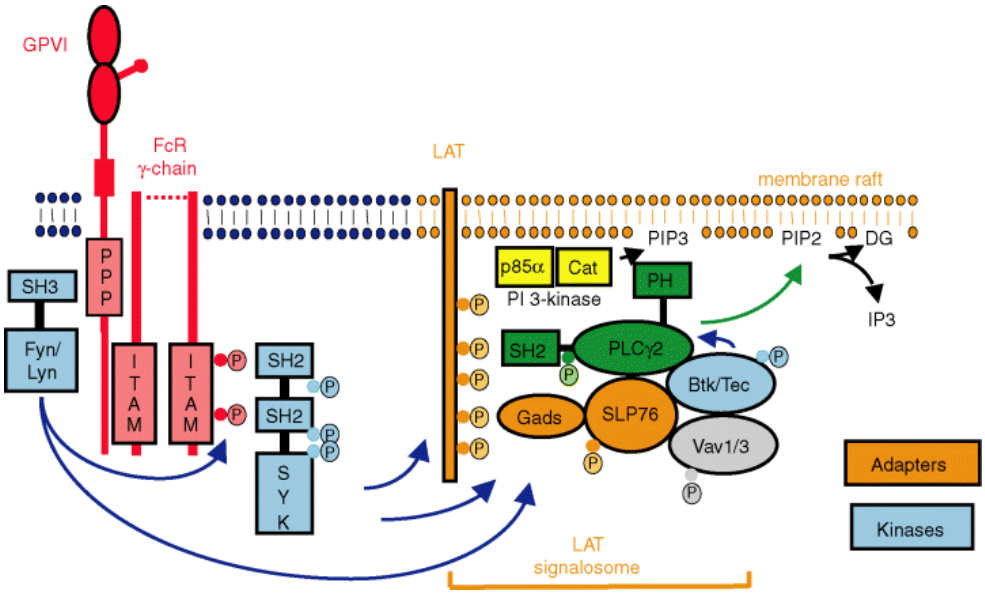


Figure 1.5: Glycoprotein VI signalling. The activation of GPVI-FcRγ complex leads to Syk dependent formation of LAT signalosome which include Gads, SLP76 and PLCγ2 isoform that enables the mobilisation of secondary messengers involved in platelet activation (Watson et al., 2005).

1.2.2.3 The role of glycoprotein VI in haemostasis

The major functional role for GPVI in haemostasis is to prevent excessive blood loss after injury. Recently, two patients have been reported with a mild bleeding phenotype due to mutations of GPVI (Dumont et al., 2009, Hermans et al., 2009). Both patients had compound heterozygous mutations preventing the expression of the receptor or causing impaired function.

Much of our understanding about the role of GPVI in haemostasis is from GPVI or FcR-γ deficient mice. These genetically engineered mice fail to undergo aggregation to collagen and exhibit defects to both adhesion and aggregation under flow (Poole et al., 1997, Best et al., 2003, Kato et al., 2003). Furthermore, FcR-γ deficient mice only show delayed and decreased thrombosis formation *in vivo* following only mild injury (Kalia et al., 2008, Senis et al., 2009). Interestingly, mice deficient in either GPVI or FcR-γ only have a mild increase in tail bleeding time and therefore may demonstrate that other mechanisms can compensate for loss of GPVI and prevent excess blood loss (Kato et al., 2003, Kalia et al., 2008).

1.2.3 Platelet secretion

During the extension phase platelets release soluble agonists such as ADP and TxA_2 that reinforce platelet activation and thrombus formation. The success of anti-thrombotic agents such as aspirin and clopidgrel to target TxA_2 synthesis and ADP signalling respectively has shown the importance of these secondary mediators in thrombus formation. The release of ADP from platelet dense-granules is a complex process that involves soluble NEM-sensitive attachment protein receptors (SNARE) and associated proteins (Chung et al., 2000, Feng et al., 2002, Barclay et al., 2003, Polgar et al., 2003). The activation of PKC and intracellular Ca^{2+} mobilisation signalling pathways are essential for granule secretion (Harper and Poole, 2007). PKC is a serine-threonine kinase which plays an important role in platelet activation, adhesion and thrombus formation (Harper and Poole, 2010). This kinase is composed of a catalytic domain attached to a regulatory domain via a hinge (Murugappan et al., 2004, Buensuceso et al., 2005, Cohen et al., 2009). Conventional PKC isoform (α and β) are expressed in human platelets and are sensitive to both the secondary messengers, Ca^{2+} and DAG. These PKC isoforms contain the Ca^{2+} sensitive domain, C2 and the DAG sensitive domain, C1 in the regulatory domain (Heemskerk et al., 2011). In contrast, the novel PKC isoforms (δ and θ) which are also expressed in human platelets can only bind to DAG and are insensitive to Ca^{2+} (Yoshioka et al., 2001). Due to lack of specific PKC isoform inhibitors, the role of these isoforms in granule secretion has been extensively investigated in genetically engineered mice. PKCα isoform-deficient mice show almost complete loss of alpha and dense-granule secretion in platelets induced by thrombin or CRP-XL stimulation (Konopatskaya et al., 2009). However, platelets from PKC δ isoform-deficient mice show enhanced GPVI-dependent secretion and decreased granule secretion in PAR-stimulated platelets (Chari et al., 2009). Therefore, PKC δ isoform can positively or negatively regulate granule secretion in an agonist dependent manner. Experiments with PKC θ isoform-deficient mice have shown that this isoform can negatively

regulate GPVI-dependent granule secretion, especially at low concentrations (Hall et al., 2008, Harper and Poole, 2009). However, reduced granule secretion has been reported at higher CRP-XL concentrations and therefore suggesting a negative and positive feedback mechanism dependent on the agonist concentration (Nagy et al., 2009).

1.2.3.1 Mechanism of platelet activation by ADP

ADP is stored in platelet dense-granules and is released upon platelet activation. Studies have shown that platelet stimulation by ADP promotes TxA_2 synthesis, shape change and aggregation (Hechler et al., 1998, Daniel et al., 1999, Kim and Kunapuli, 2011). ADP acts through two G-protein coupled receptors, $P2Y_1$ and $P2Y_{12}$ (Hollopeter et al., 2001, Kunapuli et al., 2003). The $P2Y_1$ receptor is coupled to G_q and $P2Y_{12}$ receptors are coupled to G_i α-subunits (Daniel et al., 1999, Kunapuli et al., 2003). Activation of $P2Y_1$ induces the pertussis-toxin resistant G_q to activate PLCβ isoform. G_q mediated activation of PLCβ isoforms increase IP_3 and DAG formation that enables the mobilisation of intracellular Ca^{2+}, activation of PKC and supports $\alpha_{IIb}\beta_3$ activation (Jin et al., 1998, Daniel et al., 1999). Pathways that involve the small G-protein RhoA and Src-kinases have been reported to be activated downstream of $P2Y_1$ that support TxA_2 formation and shape change (Dorsam and Kunapuli, 2004, Kahner et al., 2006). Studies on $P2Y_1$-deficient mice have shown that these platelets failed to aggregate or change shape in response to ADP-stimulation (Fabre et al., 1999). These platelets also show severely impaired aggregation in response to collagen (Fabre et al., 1999).

ADP activation of $P2Y_{12}$ induces G-protein G_i to dissociate into $G_{i\alpha}$ and $G_{\beta\gamma}$ subunits that activate different pathways. $G_{i\alpha}$ subunit is able to inhibit adenylyl cyclase and therefore decreases intracellular cAMP levels (Kunapuli et al., 2003). It is well established that high cytosolic cAMP levels inhibit platelet responsiveness to platelet activating factors (Eigenthaler et al., 1992). The dissociated $G_{\beta\gamma}$ subunit can activate PI3K isoforms that enhance granule secretion and $\alpha_{IIb}\beta_3$ activation through the pathways involving Akt and RapIb (Woulfe et al., 2002, Kim et al., 2004). These pathways are able to synergise with low doses of other agonists such as collagen to enhance platelet aggregation (Atkinson et al., 2001, Dangelmaier et al., 2001). Platelets from $P2Y_{12}$-deficient mice show a significant reduction in ADP-induced aggregation, with no effect on $P2Y_1$ receptor induced shape change (Foster et al., 2001). These mice also

exhibit an increase in tail bleeding time and therefore demonstrate the importance of this receptor in haemostasis and thrombus formation (André et al., 2003).

The release of ADP at the site of vascular injury must be regulated. The release of ADP from platelet dense-granules along with damaged red blood cells are broken down by ectonucleotidases (CD39) found in the endothelium (Boeynaems et al., 2005). This will limit ADP-mediated platelet activation and thrombus formation.

1.2.3.2 The role of thromboxane A_2 in platelet function

TxA_2 is synthesised from the hydrolysis of arachadonic acid released from phospholipids by phospholipase A_2 (PLA_2). Newly liberated arachadonic acid is converted to TxA_2 by sequential steps involving cyclo-oxygenase and TxA_2-synthase (Henriksen et al., 1997). Once formed it can easily diffuse across the plasma membrane and activate other platelets that are in close proximity. TxA_2 has a very short half-life of 30 seconds and this limits the spread of platelet activation. TxA_2 acts through two G-protein coupled receptors, TPα/β that are coupled to G_q and G_{12}/G_{13} families (Shenker et al., 1991). Similar to ADP signalling, TxA_2 signalling involves the activation of PLCβ isoforms by G_q to increase the formation of IP_3 and DAG and therefore enabling Ca^{2+} mobilisation and PKC activation (Berridge, 1993). It is now well established that TxA_2 signalling initially drives a first wave of ADP release which promotes integrin activation and aggregation (Li et al., 2003). Studies have shown TxA_2 is important in the platelet response to collagen (Nieswandt and Watson, 2003). Collagen-induced platelet aggregation is both TxA_2 and ADP-dependent, especially at lower concentrations (Atkinson et al., 2001). In contrast, thrombin-induced aggregation does not require secondary mediators (Macfarlane et al., 1975).

The TP receptor isoforms are also coupled to G_{12}/G_{13} which has been linked to platelet shape change (Klages et al., 1999, Dorsam et al., 2002, Nieswandt et al., 2002). Platelet shape change has been shown to involve the interaction between myosin and actin through the phosphorylation of myosin light chain (MLC) (Daniel et al., 1984). The phosphorylation of MLC is regulated by MLC kinase and myosin phosphatase. Platelet stimulation by TxA_2 results in the phosphorylation of the MLC and inactivation of myosin phosphatase in a Rho-kinase dependent manner (Dorsam et al., 2002, Nieswandt et al., 2002, Paul et al., 2003).

1.2.4 Thrombin

Thrombin is a potent platelet activator and can activate platelets at a very low concentration. Thrombin, a serine protease is formed locally at the site of vascular damage from the inactive precursor prothrombin (Esmon et al., 1974). The prothrombinase complex is confined to the platelet surface by the expression of negatively charged phosphatidylserine, and in this way thrombin is generated in the immediate vicinity of its site of action. Thrombin binds to G-protein coupled receptors belonging to the protease-activating receptor family, namely PAR1 and PAR4 , which are coupled to G_q and G_{12}/G_{13} (Kahn et al., 1999). Thrombin cleaves the N-terminus of these receptors which can act as a tethered ligand. In the cleaved state, part of the receptor itself acts as the agonist, causing a physiological response (Macfarlane et al., 2001). Once activated, this leads to the activation of PLCβ and the subsequent mobilisation of intracellular Ca^{2+} and PKC activation, similar to ADP and TxA_2 platelet activation mechanism described earlier. Initially thrombin signals through PAR1 and then PAR4, prolonging the duration of the thrombin response (Covic et al., 2000). Activation of G_{12}/G_{13} leads to the rearrangement of the actin cytoskeleton and shape change in a Rho and Rac dependent mechanism (McCarty et al., 2005).

1.2.5 Platelet aggregation

Platelet aggregation is essential in the formation of the haemostatic plug. The importance of platelet aggregation was first recognised in the late 19th century by Bizzozero (1882) who correctly described platelet clumping at sites of vascular injury (Bizzozero, 1882). However, it was not until the discovery of the aggregometer in 1962 by Born (Born, 1962) which allowed us to study platelet aggregation *in vitro*. This relatively simple assay was designed to assess the ability of activated platelets to clump together in suspension and the changes in light transmission could accurately measure platelet aggregation (see section 2.3.1).

Integrin $\alpha_{IIb}\beta_3$ is a highly expressed platelet surface receptor that plays a critical role in platelet aggregation and haemostasis. There are approximately 80,000-100,000 copies of $\alpha_{IIb}\beta_3$ per platelet, which accounts for 3% of total cell protein (Phillips et al., 1988). Platelet alpha-granules also contain $\alpha_{IIb}\beta_3$ and become available upon platelet activation (Cramer et al., 1990). Integrin $\alpha_{IIb}\beta_3$ is composed of two heterodimeric subunits, α_{IIb} and β_3 which are associated with each other through non-covalent interactions (Fujimura and Phillips, 1983). Successful formation of the haemostatic plug requires the activation of integrin $\alpha_{IIb}\beta_3$ from a low to high affinity state. This transition from a low to high affinity state alters the conformation of the integrin to allow the binding of extracellular ligands which include fibrinogen, vWF, fibronectin, vitronectin and thrombospondin-1 (Jackson, 2007). However, only fibrinogen and vWF directly support platelet aggregation via the interaction of $\alpha_{IIb}\beta_3$ with the arginine-glycine-serine (RGD) motif (Jackson, 2007). Initial platelet activation and signalling by G-protein coupled receptors, GPVI, GPIb-IX-V and other receptors enable the activation of $\alpha_{IIb}\beta_3$ by a process known as inside-out signalling (Shattil, 1999, Shattil and Newman, 2004). Activated integrin $\alpha_{IIb}\beta_3$ can bind to fibrinogen allowing platelets to cross-link with each other and form a stable haemostatic plug. In addition, activated integrin $\alpha_{IIb}\beta_3$ can signal once bound to a ligand in a process called outside-

in signalling (Shattil, 1999, Phillips et al., 2001). This process reinforces platelet activation and causes irreversible aggregation (Phillips et al., 2001).

Fibrinogen is a 340 kDa soluble plasma protein synthesised by the liver that plays a critical role in platelet aggregation and haemostasis (Bennett, 2001, Mosesson, 2005). Fibrinogen is composed of two sets of polypeptide chains called α, β and γ (Mosesson, 2005). Fibrinogen contains two RGD motifs in the α-chain and a non-RGD sequence in the γ-chain (Kloczewiak et al., 1984, Bennett, 2001). This Lysine-Glutamate-Aspartate-Valine sequence rather than the two RGD motifs is the most important in integrin $\alpha_{IIb}\beta_3$ binding (Springer et al., 2008). Fibrinogen has two main roles in haemostasis, (i) ligand for $\alpha_{IIb}\beta_3$ (ii) and the precursor for fibrin formation. Fibrinogen is cleaved to form fibrin by the protease action of thrombin in the coagulation cascade (Mosesson, 2005). Fibrin formation is essential for the anchoring of thrombi to the injured vessel wall. Furthermore, fibrinogen plays an essential role in the stability of the thrombus by bridging $\alpha_{IIb}\beta_3$ between neighbouring platelets (Holmback et al., 1996).

Studies with vWF or fibrinogen-deficient mice have demonstrated that vWF plays an important role in platelet aggregation under high shear (Denis et al., 1998, Ni et al., 2000). Kulkami and colleagues showed platelet aggregation under flow is a multi-step process involving the exposure of vWF (Kulkarni et al., 2000). The first step of this process is the interaction of vWF with GPIb to cause reversible aggregation. The second step was the irreversible aggregation dependent on integrin $\alpha_{IIb}\beta_3$ interaction with a ligand which was associated with platelet shape change and granule release. However, platelet aggregation and thrombus formation was observed in mice deficient in both fibrinogen and vWF, although the thrombi were very unstable (Ni et al., 2000). Recently a third mechanism initiating platelet aggregation was proposed at very high shear rate (>10,000 s^{-1}). This mechanism of platelet aggregation does not require platelet activation or integrin $\alpha_{IIb}\beta_3$ ligand binding (Ruggeri et al., 2006). In this case platelet aggregates were formed via GPIb-vWF adhesive bonds. However, it is important to remember that the platelet aggregometer can

only test the traditional mechanism of platelet aggregation involving integrin $\alpha_{IIb}\beta_3$ and fibrinogen binding.

1.3 Regulation of platelet function by cyclic nucleotides

Platelets circulate in an inactive state and are activated once they are exposed to sub-endothelial proteins which lead to the formation of a haemostatic plug. The balance between haemostasis and thrombus formation must be tightly regulated. Platelets are regulated by platelet inhibitors released from the endothelium, such as nitric oxide (NO) and prostacyclin (PGI_2). These inhibitors induce the production of platelet cyclic nucleotides which activate downstream kinases that reduce platelet activity by inhibiting key enzymes involved in platelet adhesion, shape change, degranulation and aggregation (Nishikawa et al., 1984, Halbrügge et al., 1990, Giesberts et al., 1995, Jin et al., 2005).

NO is a free radical synthesised *in vivo* from L-arginine by endothelial nitric oxide synthase (eNOS) (Alderton et al., 2001). NO is able to freely diffuse across the platelet membrane and enter the cytosol where it activates soluble guanylyl cyclase (sGC) and it converts GTP to cyclic guanosine monophosphate (cGMP) resulting in the activation of protein kinase G (PKG). PKG is a serine-threonine kinase which phosphorylates target proteins leading to inhibition of most platelet activatory pathways (Schwarz et al., 2001). PKG-deficient mice show increased platelet adhesion and aggregation *in vivo* (Massberg et al., 1999). Platelet activation by many platelet agonists depends on the mobilisation of intracellular calcium via PLC activation. It is widely accepted that NO can reduce platelet activation by inhibiting intracellular calcium mobilisation. For example, the mobilisation of intracellular calcium is restricted through the inhibition of IP_3 receptors and activation of sarcoendoplasmic reticulum calcium ATPase (Enouf et al., 1997). Furthermore, PKG has been shown to directly phosphorylate PLCβ1 and β2 isoforms and thereby reducing its activity by 70% (Xia et al., 2001). The inhibition of PLC isoforms by NO signalling pathway also limits the activation of PKC through the decreased DAG production (Jin et al., 2005). NO has also been shown to inhibit platelets via calcium independent actions. These include inhibition of PI3K, PKC, ADP

receptor $P2Y_{12}$ and MLC kinase (Nishikawa et al., 1984, Gopalakrishna et al., 1993, Pigazzi et al., 1999, Aktas et al., 2002).

The endothelium releases another potent platelet inhibitor, PGI_2 which helps to limit unwanted platelet activation. PGI_2 is able to elevate cytosolic cAMP levels through the activation of adenylyl cyclase via the G_s-coupled IP receptors (Gorman et al., 1977). Elevated cAMP levels activate protein kinase A (PKA), a serine-threonine kinase which also targets calcium mobilisation and PLC activation pathways as previously described for NO. The release of platelet derived TxA_2 is an important event in platelet activation and thrombus formation. Activation of PGI_2/PKA pathway has been demonstrated to inhibit TxA_2 secretion and signalling by the phosphorylation of serine^{329} within the carboxyl terminal of the TPα receptor (Walsh and Kinsella, 2000). This phosphorylation disrupts the coupling of the receptor to G_q and thereby limiting its activation (Walsh and Kinsella, 2000). PGI_2 has also been shown to limit integrin $\alpha_{IIb}\beta_3$ dependent aggregation by returning the integrin to its low affinity state and therefore preventing fibrinogen binding (Giesberts et al., 1995). The action of NO and PGI_2 normally work synergistically to regulate platelet function.

1.4 Adipokines

Cardiovascular disease is a major cause of death in the western world (Naseem, 2005). Obesity is a major risk factor in developing cardiovascular disease along with metabolic abnormalities such as hyperglycaemia and hyperlipidaemia (Grundy, 2004, Aggoun, 2007). It is widely known that obesity is often the cause of the insulin resistance that leads to these metabolic abnormalities (Ferrannini et al., 1997). Adipose tissue has been widely recognised as a complex endocrine organ that plays a central role in energy and vascular homeostasis (Kershaw and Flier, 2004). In addition to its role as an energy reserve, adipose tissue also secretes metabolic hormones, enzymes, anti-fibrinolytic proteins and inflammatory cytokines (Ahima and Flier, 2000, Kershaw and Flier, 2004). These secretory proteins are called adipokines which include leptin, tumour necrosis factor-alpha (TNF-α), plasminogen activator inhibitor type 1 (PAI-1), resistin and adiponectin [Table 1.3]. Adiponectin and leptin are the major adipokines released from adipose tissue which participate in the regulation of energy homeostasis and are the major insulin-sensitising hormones (Combs et al., 2003). Leptin stimulates fatty acid oxidation, glucose uptake and prevents lipid accumulation in adipose and non-adipose tissues (Kamohara et al., 1997). In contrast, a pro-inflammatory adipokine, resistin has the opposite effect to that of leptin and adiponectin. Circulating levels of resistin are elevated in obesity and can cause insulin resistance (Degawa-Yamauchi et al., 2003). In fact, many pro-inflammatory adipokines such as retinol binding protein 4 (RBP4) in circulation are elevated in obesity and type II diabetes which contributes to the development of cardiovascular disease (Yang et al., 2005). These pro-inflammatory adipokines induce the expression of cell adhesion molecules such as intracellular adhesion molecule (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1) and E-selectin which promote vascular inflammation and the development of atherosclerosis (Ahima and Flier, 2000). However, some adipokines such as adiponectin have been described to have anti-inflammatory and anti-artherogenic properties (Okamoto et al., 2002, Ouchi and Walsh, 2007, Zhu

et al., 2008). In this section we will discuss the function of adiponectin in metabolism and the vascular system and in particular explore possible platelet-adiponectin interaction.

Adipokine	Concentration in circulation	Primary function
Leptin	2-10 ng/ml	Metabolic homeostasis
TNF-α	5-10 pg/ml	Inflammation
PAI-1	10-20 ng/ml	Haemostasis
Resistin	5-15 ng/ml	Metabolic homeostasis
RBP4	5-20 μg/ml	Retinol binding protein/ metabolic homeostasis
Adiponectin	5-30 μg/ml	Metabolic homeostasis

Table 1.3: Summary of adipokines derived from adipose tissue. A wide range of adipokines are released from adipose tissue that plays an important role in metabolic and vascular homeostasis.

1.5 Adiponectin

Adiponectin was originally discovered by four research groups (Scherer et al., 1995, Hu et al., 1996, Maeda et al., 1996, Nakano et al., 1996). In 1995, Scherer and colleagues identified a protein by cDNA cloning from the mouse adipocyte cell line 3T3-L1 and named it adipose complement related protein (Acrp30) (Scherer et al., 1995). Hu and colleagues (1996) using the same cell line isolated the same protein and called it AdipoQ (Hu et al., 1996). Nakano and colleagues isolated the protein by high affinity chromatography and called it gelatine binding protein 28 kDa (GBP-28) (Nakano et al., 1996). The name adiponectin was proposed by Arita and colleagues, in 1998 but all four names are used (Kihara et al., 1998). In this thesis the most common name of the protein, adiponectin will be used.

1.5.1 Synthesis and structure of adiponectin

Adiponectin is a 244 amino acid protein synthesised by adipocytes, most abundant gene transcript (ApM1) found on chromosome 3 locus 3q27 in adipose tissue (Hu et al., 1996). This locus is of special interest since it is associated with increased susceptibility to type II diabetes and cardiovascular disease (Kankova et al., 2007). ApM1 consists of three exons and two introns which shares similarity with leptin encoding gene (Saito et al., 1999). Adiponectin synthesis is regulated by several mechanisms and studies have shown that insulin-like growth factor-1 (IGF-1) stimulates adiponectin gene expression in adipose tissue (Berg et al., 2002). Members of the nuclear hormone family, peroxisome proliferator activated receptors (PPAR) are also involved in the regulation of adiponectin synthesis (Maeda et al., 2001). Recently, some studies have shown that other tissues may express adiponectin such as bone (Berner et al., 2004), mammary glands (Weyermann et al., 2006), salivary glands (Katsiougiannis et al., 2006) and cardiac tissue (Pineiro et al., 2005) albeit in very limited quantities**.**

1.5.1.1 Structure of adiponectin

Adiponectin, a single monomer is composed of four structurally distinct domains, an amino terminal sequence, a variable region, a collagen-like domain and an amino terminal globular domain [Figure 1.7] (Kadowaki and Yamauchi, 2005); this is termed as full-length adiponectin (fAd). Adiponectin is structurally similar to complement C1q protein (Scherer et al., 1995) and the globular fragment is structurally similar to TNF-α (Berg et al., 2002). C1q and TNF-α both play an important role in inflammation and the immune response. Circulating adiponectin is found primarily as disulfide-linked oligomers, composed of trimers, hexamers and a high molecular mass multimers (HMW), containing up to 18 monomers [Figure 1.8]. Adiponectin trimers are generated when a triple helix is formed by non-covalent interactions between the collagenous domains and hydrophobic interactions between the globular head groups (Scherer et al., 1995, Shapiro and Scherer, 1998). Oligomer formation of adiponectin is dependent on the disulfide bond formation mediated by cysteine 39 (Pajvani et al., 2003). Furthermore, biochemical analysis of purified complexes and *in vivo* studies have revealed that different forms of adiponectin complexes do not interconvert after secretion (Simpson and Whitehead, 2010).

Several years after the initial discovery of adiponectin, a globular form of protein was identified in the circulation of mice (Kishida et al., 2003) and humans (Fruebis et al., 2001). This form contains the globular head but is deficient in the collagen-like domain. Without the collagen-like domain, the globular domain of adiponectin still trimerises, but does not associate into HMW isoforms (Waki et al., 2005). It is thought that both the globular and the collagenous domains are important for ensuring the stability and activity of the multimeric forms. Nearly all circulating plasma adiponectin exists as full-length multimeric forms of adiponectin. The source of gAd is unknown, it has been demonstrated *in vitro* that leukocyte elastase secreted from activated human monocytes and neutrophils was responsible for the proteolytic cleavage of recombinant murine fAd to form

the globular fragment (Waki et al., 2005). However, the physiological importance of this remains unclear.

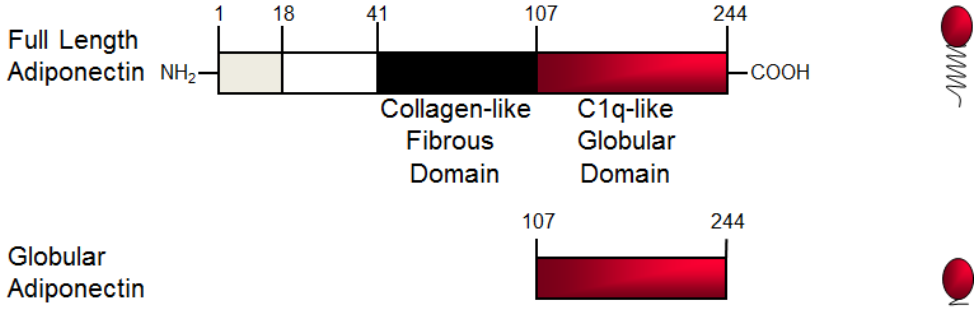


Figure 1.7: Structure of different isoforms of adiponectin. FAd consists of three main domains; a variable domain, collagen-like domain and globular domain. The globular domain can be cleaved to form the gAd isoform.

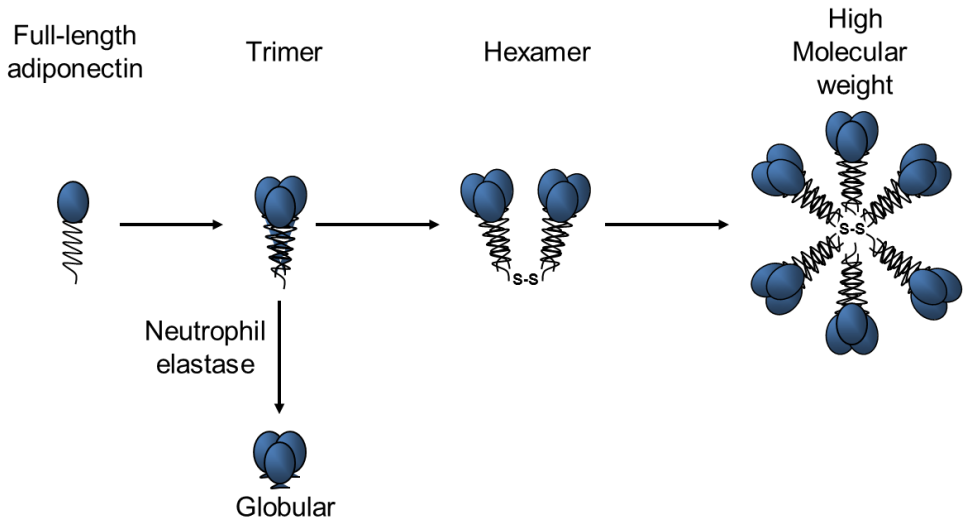


Figure 1.8: Multiple forms of adiponectin in circulation. FAd monomers consist of a globular head and a collagen triple helix which can trimerise through the formation of disulfide bonds. Adiponectin trimers can further combine to form hexamers and high molecular weight isoforms. However, the globular head can be cleaved by neutrophil elastase to form gAd.

1.5.1.2 Synthesis of adiponectin

The molecular details of adiponectin synthesis pathway remains poorly defined. Recent studies with murine derived fAd have shown that chaperone proteins such as BiP, ERp44, Ero1-Lα and Dslb aid the assembly of adiponectin multimers and secretion *in vitro* (Qiang et al., 2007, Wang et al., 2007, Liu et al., 2008, Simpson and Whitehead, 2010). Furthermore, high levels of expression of these chaperones result in efficient adiponectin secretion and are associated with high circulating adiponectin levels. The secretion of adiponectin is regulated by the binding of adiponectin in the endoplasmic reticulum (ER) to the protein ERp44 through a thiol bond with cysteine 39, therefore adiponectin is retained in the ER (Wang and Scherer, 2008). It is secreted when ERp44 forms a new thiol bond with a second protein Ero1-Lα, thus releasing the previous thiol-bound adiponectin (Anelli et al., 2003). The treatment of cells with β-mercaptoethanol breaks this bond to increase the secretion of these isoforms into the culture medium *in vitro* (Anelli et al., 2003). Once synthesised, adiponectin undergoes extensive post-translational hydroxylation and glycosylation modifications to produce eight isoforms. Six of the adiponectin isoforms are glycosylated at four lysine residues, 68, 71, 80 and 104. A proline residue 94 located within the collagenous domain is also glycosylated (Wang et al., 2008). The secretion of adiponectin is dependent on Gamma-ear adaptor 1 coated vesicles (Xie et al., 2006). This field of work has attracted a lot of interest lately since adiponectin synthesis is down regulated in disease and therefore, understanding the molecular mechanism of adiponectin synthesis can have a potential therapeutic value.

1.5.2 Adiponectin in circulation

Adiponectin circulates in the plasma at approximately 5-30 μg/ml, accounting for 0.01% of the total plasma protein (Arita et al., 1999). This concentration is higher than that of most hormones found in the circulation, for example leptin and cortisol circulate in ng/ml, while TNF-α or interleukin-6 are measured in pg/ml. Furthermore, eight human serum proteins have been identified that were co-purified with adiponectin. Many of these proteins include thrombospondin-1, histidine-rich glycoprotein, kinogen1 and alpha 2 macroglobulins. These proteins might bind directly or indirectly to adiponectin in the circulation and may modulate adiponectins anti-inflammatory and anti-artherogenic activities (Wang et al., 2006). Furthermore, albumin has been found to be associated with the low molecular weight isoforms of adiponectin through the formation of a disulfide bond (Hada et al., 2007).

1.5.3 Adiponectin receptors

There are two distinct adiponectin receptors identified, called AdipoR1 and AdipoR2, which are thought to facilitate the biological actions of adiponectin (Yamauchi et al., 2003). Northern blot analysis of human and murine tissue found mRNA from AdipoR1 was abundantly expressed in skeletal muscle and to a lesser extent in the heart and spleen. In contrast, AdipoR2 expression was mostly restricted to the liver (Yamauchi et al., 2003). The expression of these adiponectin receptors have been identified in monocytes (mRNA and protein) and are thought to play an important role in the immune system (Weigert et al., 2008). The existence of these adiponectin receptors in platelets is not clear. Kato and colleagues have found AdipoR1 and AdipoR2 (mRNA) expressed in murine platelets (Kato et al., 2006). Furthermore, AdipoR1 (mRNA) was detected in human platelets, whereas both AdipoR1 and AdipoR2 were found in megakaryocyte cell lines (Kato et al., 2006). Analysis of AdipoR1 and AdipoR2 has shown that AdipoR1 has a very strong affinity for gAd and AdipoR2 is the preferred receptor for fAd (Kadowaki and Yamauchi, 2005).

AdipoR1 and AdipoR2 are composed of seven transmembrane domains similar to that of G-protein coupled receptors. However, AdipoR1 and AdipoR2 do not transduce G-protein coupled signalling [Figure 1.9] (Yamauchi et al., 2003). The N-terminus of AdipoR1 and AdipoR2 are found in the cytoplasmic region of the cell and the C terminus is found externally which is opposite to that of G-protein coupled receptors. Once activated AdipoR1 and AdipoR2 can form both homo and heterodimeric complexes, however, the physiological significance is unknown. In 2004, a third receptor for adiponectin was proposed, T-cadherin, a member of the cadherin superfamily which are involved in cell adhesion and signalling. T-cadherin is thought to bind to hexamers and HMW adiponectin oligomers (Hug et al., 2004). The existence of these adiponectin receptors can be explained by different isoforms of adiponectin which require different receptor conformations to ensure a high binding affinity, and by the wide biological actions of adiponectin in different tissues.

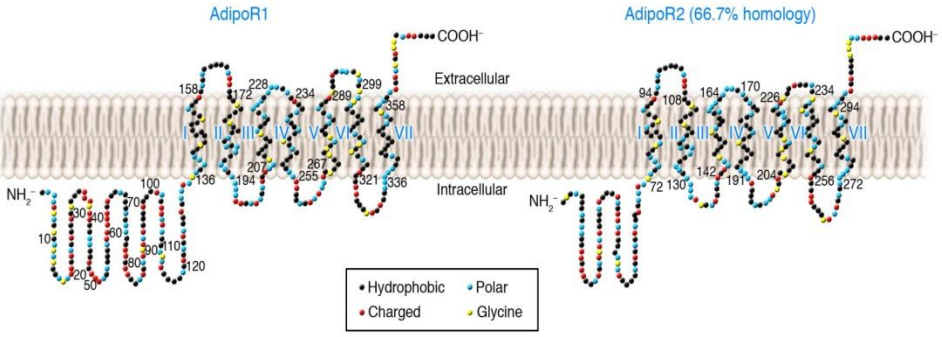


Figure 1.9: Adiponectin receptors. Adiponectin signals through two G-protein like receptors, AdipoR1 and AdipoR2 which consist of seven transmembrane domains (Kadowaki and Yamauchi, 2005).

1.5.4 Insulin-sensitising actions of adiponectin

The insulin-sensitising effect of adiponectin was first identified by 3 independent groups (Berg et al., 2001, Fruebis et al., 2001, Yamauchi et al., 2001). Berg and colleagues showed *in vivo* that fAd decreased basal glucose levels by inhibiting the expression of hepatic gluconeogenic enzymes, such as phosphoenolpyruvate carboxylase and glucose-6-phosphatase (Berg et al., 2001). Similar data was obtained by Yamauchi and colleagues and suggested a link between adiponectin and insulin resistance (Yablonski et al., 2001). Fruebis and colleagues showed that gAd was involved in lipid metabolism (Fruebis et al., 2001) and therefore these studies indicate that both forms of adiponectin are involved in metabolic homeostasis.

It has become clear that adiponectin can exert its metabolic effect through several pathways in different tissues. For example, in skeletal muscle adiponectin increases expression of CD36 which is involved in fatty acid transport and increases enzymes involved in fatty acid oxidation such as acyl coenzyme A oxidase (Tomas et al., 2002). The combined effect of increased fatty acid transport and oxidation results in a lower tissue triglyceride content. Reduced tissue triglyceride content has been shown to improve insulin signal transduction pathways since high tissue triglyceride content interferes with insulin-stimulated PI3K activation, glucose transporter-4 translocation and glucose uptake which can lead to insulin resistance (Maeda et al., 2002). Furthermore, gAd can indirectly increase fatty-acid oxidation by PPARα activation, which can decrease triglyceride content in the liver and skeletal muscle (Yamauchi et al., 2003). Adiponectin knock-out mice fed on a high fat diet developed severe insulin resistance, compared to wild-type controls (Maeda et al., 2002) and therefore clearly placing this adipokine as a central factor in metabolic health.

The metabolic effects of adiponectin are mainly dependent on its ability to activate 5' adenosine monophosphate-activated protein kinase (AMPK) enzyme which is involved in energy homeostasis. AMPK is a heterotrimeric complex composed of a catalytic α-subunit and the regulatory β and γ-subunits (Dugan et al., 1982). Both gAd and fAd can stimulate AMPK activation in skeletal muscle, whereas only fAd activates AMPK in the liver (Combs et al., 2003). Experimentally, gAd has been shown to activate AMPK in primary rat adipocytes to increase glucose uptake (Wu et al., 2003). Therefore, both gAd and fAd exerts their metabolic effects through different pathways as shown in Figure 1.10.

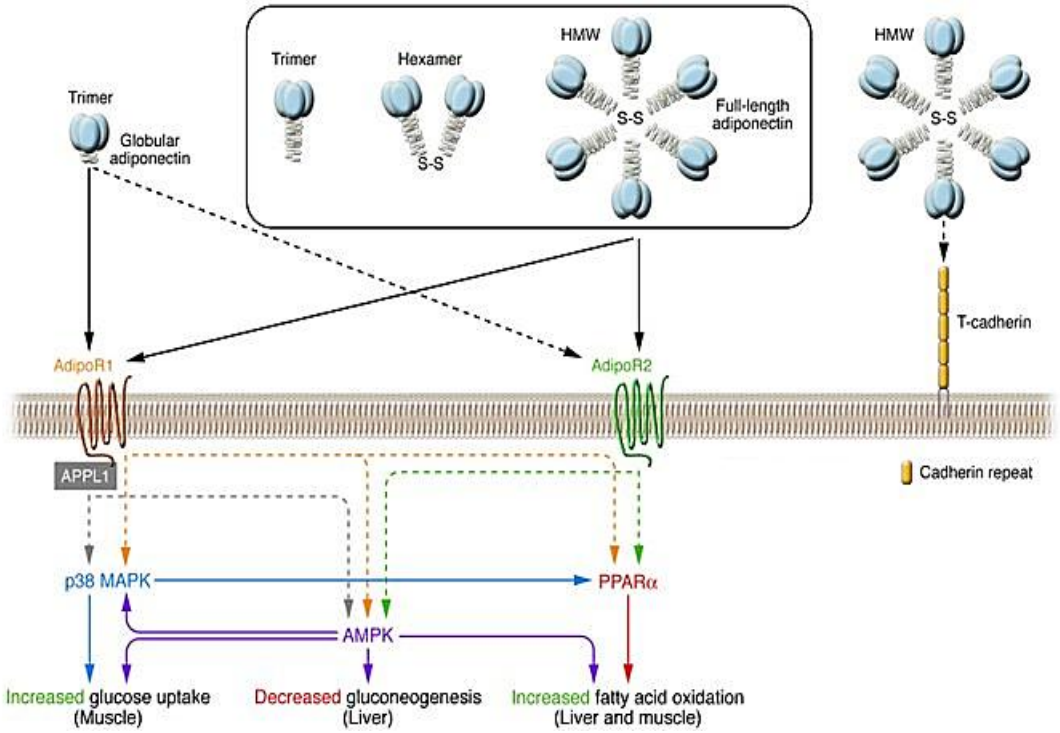


Figure 1.10: Metabolic effects of adiponectin. AdipoR1 and AdipoR2-mediated activation by different adiponectin isoforms stimulate glucose uptake and fatty acid oxidation through the activation of AMPK and p38 mitogen-activated protein kinase (MAPK), which results in increased insulin sensitivity (Kadowaki et al., 2006).

1.5.5 Role of adiponectin beyond vascular biology

A number of recent studies in humans and animals have demonstrated that a decrease in plasma concentrations of adiponectin increases the risk of developing type II diabetes, hypertension and cardiovascular disease (Weyer et al., 2001, Pischon et al., 2004, Kadowaki et al., 2006). Furthermore, adiponectin appears to play an important role in vascular biology. Adiponectin knock-out mice develop increased neointimal thickening and increased proliferation of smooth muscle cells compared to control mice (Matsuda et al., 2002). Importantly, adiponectin treatment reversed many of these vascular changes (Kihara et al., 1998, Ouchi and Walsh, 2007). Thus, there is clear evidence that adiponectin exerts much broader biochemical and cellular effects than simply regulating metabolism.

1.5.5.1 Atherosclerosis

Atherosclerosis is characterised by chronic systemic inflammation and the narrowing of the lumen of the blood vessel through the deposition of lipids (Ross, 1993). Studies have shown that adiponectin has many anti-atherosclerotic and cardio-protective properties. It is proposed that the anti-atherosclerotic properties of adiponectin are due to its anti-inflammatory properties. Circulating plasma adiponectin can bind to exposed extracellular matrix proteins such as collagen fibres in the damaged intima of blood vessels and subsequently can inhibit the expression of adhesion molecules, including ICAM-1, VCAM-1, E-selectin and macrophage induced TNF-α production (Okamoto et al., 2000). The inhibition of TNF-α mediated signalling and synthesis by adiponectin, through the inhibition of NF-ķB pathway in endothelial cells, prevents the expression of cell surface adhesion proteins (Ouchi and Walsh, 2007). It is known that adiponectin promotes cAMP/PKA signalling in endothelial cells, which inhibits the NF-ķB pathway (Ajuwon and Spurlock, 2005). Therefore, the activation of cAMP/PKA signalling is an important mechanism by which adiponectin protects endothelial cells from activation (Ouchi et al., 2000).

As previously described in section 1.3 the endothelium release platelet inhibitors such as NO and PGI_2 to regulate platelet function. However, the release of these inhibitors has much more broader implications in vascular disease beyond their effect on platelets (Naseem, 2005). The decreased synthesis and bioavailability of NO has been implicated in the development of atherosclerosis (Cooke and Dzau, 1997). NO can reduce leukocyte recruitment to sites of vascular inflammation and limit smooth muscle proliferation (Naseem, 2005). NO is a free radical synthesised *in vivo* from L-arginine by eNOS (Alderton et al., 2001). The production of NO can be up-regulated by adiponectin through the phosphorylation of eNOS at serine^{1777} by pathways requiring AMPK activation (Chen et al., 2003). Adiponectin can also increase the formation of eNOS and heat shock protein 90 complex which is necessary for NO synthesis (Xi et al., 2005).

The accumulation of lipid-laden foam cells and macrophage related inflammation is an important stage in the development of atherosclerosis. Adiponectin inhibits the expression of macrophage scavenger receptor class A-1, resulting in the decreased uptake of oxidised low density lipoproteins by macrophages and thus inhibiting the formation of foam cells (Ouchi et al., 2001). Decrease in plasma adiponectin has been demonstrated to increase platelet-monocyte interaction and thereby promoting the formation of the atherosclerotic plaque (Ouchi and Walsh, 2007). Taken together, adiponectin reduces atherosclerosis by reducing vascular inflammation, increasing NO synthesis and inhibiting macrophage to foam cell transformation and lipid uptake.

1.5.5.2 Pro-inflammatory properties of globular adiponectin

Anti-inflammatory properties of adiponectin are well characterised but the role of gAd in inflammation is less clear. There is emerging evidence that suggests that gAd has pro-inflammatory properties. Studies have shown that gAd induces the production of pro-inflammatory cytokines from macrophages such as IL-1β, IL-6, IL-8 and TNF-α which act to increase the expression of cell adhesion molecules and thereby promoting inflammation (Tsatsanis et al., 2005, Tsatsanis et al., 2006). Consistent with these pro-inflammatory properties, gAd has been shown to increase angiotensin-II induced proliferation of cardiac fibroblasts (Hattori et al., 2006, Hattori et al., 2007) and stimulate THP-1 production by monocytes (Neumeier et al., 2006). Thus emerging evidence suggests that gAd may possess pro-inflammatory properties in addition to its insulin-sensitising effects (Hattori et al., 2006, Hattori et al., 2007). However, the importance of these pro-inflammatory properties in disease needs further investigation.

1.5.5.3 Adiponectin and platelet function

The data linking hypoadiponectinaemia and increased platelet-monocyte interactions (Shoji et al., 2006) points to a potential effect on platelet function, although it is unclear if this is a direct effect on platelet function. Elbatarny and colleagues showed that fAd had no effect on platelet aggregation or adhesion *in vitro* (Elbatarny et al., 2007). However, it is important to note that a very low concentration of fAd was used, 0-500 ng/ml compared to 5-30 μg/ml found at physiological levels.

The major evidence for the effect of adiponectin on platelet function comes from an important study by Kato and colleagues (Kato et al., 2006). This study used a He-Ne laser–induced injury model to assess thrombus formation in adiponectin-deficient mice and proposed adiponectin as an endogenous anti-thrombotic factor. These mice displayed enhanced thrombus formation in response to injury to the carotid artery, which could be delayed by the addition of exogenous adiponectin. Analysis of platelets *in vitro* from the same adiponectin-deficient mice displayed enhanced aggregation and thrombus formation under flow conditions. However, it was unclear if the increased thrombus formation in adiponectin-deficient mice was due to the direct effect of adiponectin on platelets or the indirect effect via the vascular system. Furthermore, circulating levels of adiponectin are decreased in individuals with coronary artery disease and may promote increased risk of thrombosis (Shibata et al., 2005). Interestingly, the direct effect of gAd on platelet function has not been investigated and the effect of adiponectin on platelets may provide a tangible link between metabolic syndrome and cardiovascular disease.

1.6 Aims of thesis

In the present study the effects of adiponectin on platelet function *in vitro* was investigated. We wished to

i. Examine if different forms of adiponectin had differential effects on platelet function

ii. Determine the signalling events underlying any observed effects on aggregation

iii. Identify the receptor(s) responsible for the effects of adiponectin on platelet function

Chapter 2: Materials and methods

2.1 Materials

Recombinant human globular and full-length adiponectin was purchased from BioVision Research Products (CA, USA). Horm collagen type I (equine) was from Nycomed (Munich, Germany). Ro 31-8220, wortmannin, U46619, SQ29548, PP1 and PP3 were from Calbiochem (Darmstadt, Germany). Adrenaline, ADP, thrombin, fibrinogen, indomethacin, apyrase, MRS2395 and A3P5P were from Sigma (Poole, UK). TGX-221, PIK75 and AS242525 were from Cayman chemicals (Michigan, USA). Anti-phosphotyrosine monoclonal antibody (mAb) 4G10 antibody and anti-β-tubulin mAb were from Upstate Biotechnology (Milton Keynes, UK). Anti-PKC substrate mAb recognising the sequence (R/K)X(S*)(Hyd)(R/K) and anti-Akt serine^{473} mAb were from New England Biolabs (Hertfordshire, UK). CRP-XL and GPVI blocking antibody were from Prof. Richard Farndale, University of Cambridge (UK). The integrin $\alpha_2\beta_1$ blocking antibody 6F1 was a kind gift from Prof. Barry Coller, The Rockefeller University (USA). The chrono-lume reagent was from Labmedics (Manchester, UK).

2.2 Methodology for the study of platelets

2.2.1 Isolation of human platelets

Human blood was collected from volunteers by a trained phlebotomist and written consent was obtained from each volunteer. The consent form was used in conjunction with a short health questionnaire used to collect information about the volunteers health status, i.e. the presence of any transmitted infections and the use of prescribed and over the counter medication. In particular, the volunteers were asked about the use of aspirin or any other drugs that could interfere with platelet function.

Human blood was collected into pre-warmed acid citrate dextrose (ACD; 29.9 mM sodium citrate, 113.8 mM glucose, 72.6 mM sodium chloride and 2.9 mM citric acid, pH 6.4) at a ratio 1:6. ACD chelates calcium from the blood and thus preventing the coagulation cascade, and lowers the pH of the blood to 6.5 and subsequently inhibiting platelet aggregation (Mustard et al., 1972, Cazenave et al., 2004). The first 5 ml of venous blood collected from the volunteer was discarded as the platelets possibly could be partially activated due to the procedure. The citrated blood was gently inverted to ensure equal mixing with the ACD and was centrifuged at 120g, 20°C for 20 minutes. Centrifugation separated the blood into three layers; a well-defined bottom layer containing white and red blood cells, an intermediate layer called the buffy coat which contains substances derived from red blood cells such as ADP and the upper most layer called platelet rich plasma (PRP). Washed platelets were prepared by carefully transferring the PRP into a clean falcon tube, ensuring no contamination with the buffy coat and was further centrifuged at 800g, 20°C for 12 minutes in the presence of prostaglandin E_1 (50 ng/ml). Prostaglandin inhibits platelet activation during centrifugation and re-suspension steps by elevating cAMP levels and therefore preventing calcium mobilisation and platelet activation (Vargas et al., 1982). The platelet pellet was re-suspended at a concentration of $2.5x10^8$ platelets/ml in modified Tyrode buffer (150 mM NaCl, 5 mM HEPES, 0.55 mM anhydrous NaH_2PO_4, 7 mM

anhydrous $NaHCO_3$, 2.7 mM KCl, 0.5 mM $MgCl_2$ and 5.6 mM anhydrous glucose, pH 7.4) unless otherwise stated. The platelets were left to recover from the prostaglandin effect for one hour before use.

2.2.2 Platelet count

The platelet concentration was calculated after re-suspending the platelet pellet in modified Tyrodes buffer. Washed platelets (5 µl) were diluted 1:100 in ammonium oxalate (1% w/v) to fix the platelets and was then mixed and applied to a double chamber haemocytometer with a coverslip. The haemocytometer was left to rest for 10 minutes to allow the platelets to settle and an inverted light microscope 40x objective lens was used to count the number of platelets in the ten small squares on each side of the haemocytometer [Figure 2.1]. These ten squares have a total volume of 0.04 µl and from this the platelet count could be calculated in platelets/ml. The total number of platelets counted was multiplied by 25,000 (1000/0.04) and followed by 100 to account for the dilution with ammonium oxalate. The washed platelets volume was adjusted with modified Tyrode buffer to give the platelet count required for those experiments.

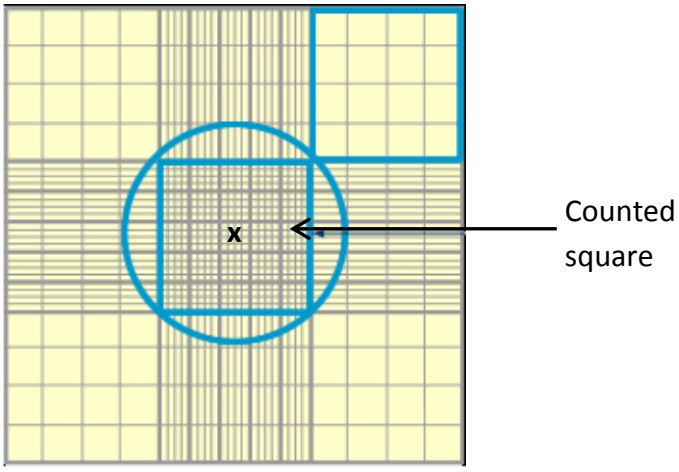


Figure 2.1: A schematic diagram of Nebauer counting chamber. Platelets were counted in the squares labelled "x".

2.3 Platelet aggregation and luminescence studies

2.3.1 Platelet aggregation

Measurement of platelet aggregation is based on a turbidimetric assay first described by Born (Born, 1962) in which changes of light transmission through a platelet suspension is detected by a photocell. In unstimulated platelet suspension light transmission is low and upon addition of platelet agonist such as collagen, the platelets undergo shape change and aggregate, thus increasing light transmission. Therefore the increase in platelet aggregation is directly proportional to increase in light transmission. These changes in light transmission can be expressed as a digital image as shown in Figure 2.2. The aggregation response can be separated into three distinct phases; platelet shape change, reversible primary aggregation where platelets can disaggregate and return back to resting state, and if a strong agonist is used, an irreversible secondary aggregation response is seen which is driven by platelet secretion.

Aggregation tubes containing 250 µl of washed platelets ($2.5x10^8$ platelets/ml) were incubated for 5 minutes at 37°C in a multi-channel Chronolog aggregometer (Pennsylvania, USA) prior to stimulation to allow for temperature equilibration. The platelet suspension were then stimulated with platelet agonists under stirring conditions and platelet aggregation was recorded for 4 minutes and expressed as percentage increase in light transmission. The platelet aggregometer was recalibrated for every individual platelet sample used. The aggregometer was calibrated using washed platelets as 0% aggregation and modified Tyrode buffer as 100% aggregation. Data was expressed as mean %aggregation ± standard error of mean (SEM) and statistical analysis was performed using the students *t* test.

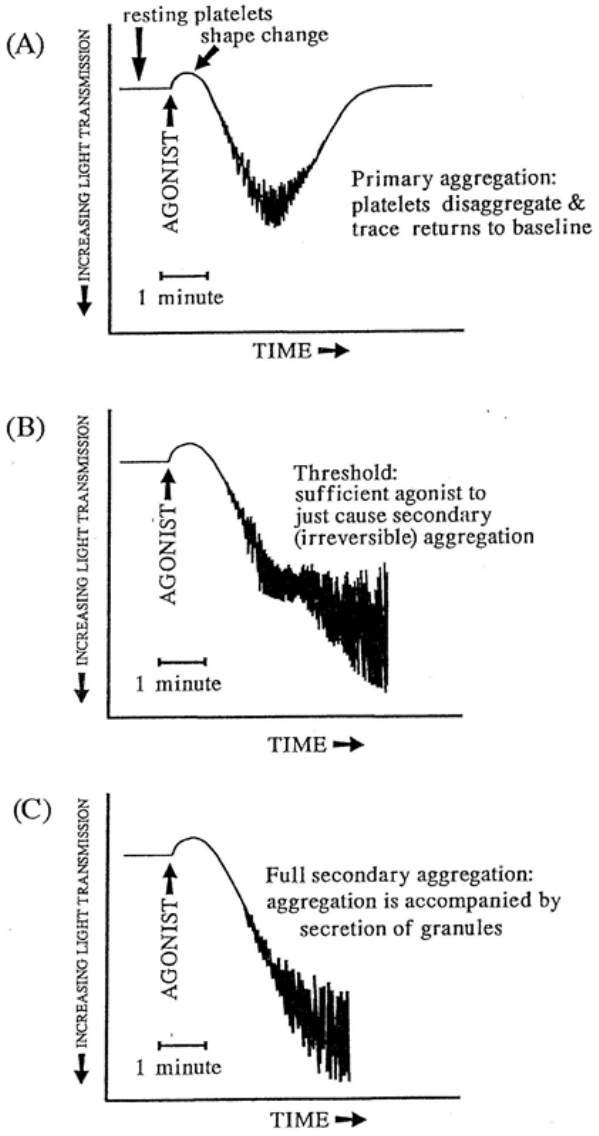


Figure 2.2: Aggregation assay. (**A**) Reversible primary aggregation characterised by shape change (**B**) Primary aggregation with some granule secretion (**C**) Full secondary aggregation response induced by granule secretion.

For some experiments platelets were incubated with pharmaceutical agents that targeted particular receptors and signalling enzymes. The incubation times used for inhibitors added prior to stimulation of platelets are given in Table 2.1.

Inhibitor	Target	Concentration	Incubation time (min)
A3P5P	$P2Y_1$	300 µM	1
MRS2395	$P2Y_{12}$	10 µM	1
Indomethacin	COX-1	10 µM	5
SQ29548	TPα/β	10 µM	5
Ro 31-8220	Pan PKC isoform inhibitor	10 µM	5
PP1	Pan Src-kinase inhibitor	10 µM	5
PP3	Structural inactive analogue of PP1	10 µM	5
Wortmannin	Pan PI3K inhibitor	100 nM	20
PIK75	PI3Kα	100 nM	20
TGX-221	PI3Kβ	500 nM	20
AS242525	PI3Kγ	100 nM	20

Table 2.1: Summary of inhibitors used in this study.

2.3.2 Measurement of ATP secretion

As highlighted in section 1.1.3, platelets release a number of factors that can enhance the aggregation response. Several methodologies have been developed to assess granule release including the measurement of P-selectin expression as a marker of alpha-granule release and ATP secretion as a marker of dense-granule release (Feinman et al., 1977, Stenberg et al., 1985). In the present study we examined aggregation simultaneously with ATP release. For these luminescence studies, platelet aggregation was performed in the presence of a luciferin-luciferase reagent and the subsequent change in luminescence was monitored in a single-channel Chrono-log lumi-aggregometer (Pennsylvania, USA) (Feinman et al., 1977). ATP released from platelet dense-granules is hydrolysed when magnesium dependent-luciferase catalyses the oxidation of luciferin into adenyl-luciferin (Reaction 1) and light is emitted with the oxidation of adenyl-luciferin into oxyluciferin (Reaction 2) [Figure 2.3]. When ATP is the limiting reagent, the light emitted is proportional to the ATP released from platelet dense-granules.

Reaction 1: Luciferin + ATP $\xrightarrow{\text{Luciferase, } Mg^{2+}}$ `Adenyl-luciferin + PPi

Reaction 2: Adenyl-luciferin + O_2 $\xrightarrow{\text{Luminescence}}$ Oxyluciferin+ H_2O + CO_2

Figure 2.3: Chemical basis of luminescence reaction.

Aggregation tubes containing 225 µl of washed platelets ($3x10^8$ platelets/ml) were incubated for 5 minutes at 37°C in the lumi-aggregometer prior to stimulation. 25 µl of luciferin-luciferase reagent was added into the washed platelets under stirring conditions for 1 minute and then the platelet suspension was stimulated with various platelet agonists and the ATP secretion was recorded for 4 minutes and data was expressed as mean ATP nM ± SEM. Statistical analysis was performed using the student *t* test. The lumi-aggregometer was calibrated with 1 nM ATP standard.

2.4 Platelet adhesion methodologies

The exposure of components of the extracellular matrix is an important event in the formation of the haemostatic plug. Initial experiments were designed to assess platelet adhesion to immobilised gAd under static and flow conditions since plasma adiponectin has been found immobilised to collagen fibres (Okamoto et al., 2002).

2.4.1 Visualisation of platelet actin cytoskeleton

Platelet adhesion to immobilised gAd under static conditions was used to assess platelet adhesion and spreading. This method relies on staining of the actin cytoskeleton with a fluorescent dye and fluorescence microscopy to visualise the platelets after they have been immobilised. Microscope slides were coated with either collagen 10 μg/ml diluted in 0.9% NaCl, CRP-XL 10 μg/ml, diluted in acetic acid (0.01M) and gAd 50 μg/ml, diluted in 0.9% NaCl or human serum (HS) (100%) overnight at 4°C. HS was obtained from the Blood Transfusion Unit (Sheffield) and prepared by heating at 80°C for 1 hour and was filtered, aliquoted and stored at -20°C. Slides were washed with modified phosphate buffered saline (PBS; 2.7 mM KCl, 1.2 mM, KH_2PO_4, 0.2 mM $MgCl_2$, 15 mM NaCl, 7.4 mM NaH_2PO_4, pH 7.4) and blocked with 5% HS diluted in 0.9% NaCl for 30 minutes at room temperature. Slides were then washed 3 times with PBS to remove any unbound protein.

Washed platelets ($5x10^7$ platelets/ml) were pre-incubated with tirofoban (2 ng/ml) for 20 minutes, an integrin $\alpha_{IIb}\beta_3$ blocking antibody to stimulate non-aggregatory conditions and with various inhibitors before adhesion to the slides for 1 hour at 37°C. Slides were then washed with PBS to ensure any unbound platelets were washed off. The adherent platelets were fixed in 4% formaldehyde for 30 minutes and washed extensively before permeabilised with 0.1% Triton X for 7 minutes. Adherent platelets were stained with TRITC-phalloidin (10 μg/ml) for 1 hour which binds specifically to the actin filaments of the adherent platelets cytoskeleton (Vandekerckhove et al., 1985). The slides were washed extensively to

ensure removal of any excess stain and histomount with a coverslip was added. The slides were visualised at x100 magnification with a fluorescent microscope (Olympus). Adherent platelets were counted from 8 random fields, an area equivalent to 0.1 mm^2 and expressed as mean number of adhered platelets ± SEM. Statistical analysis was performed using the student *t* test.

2.4.2 Visualisation of platelet P-selectin expression on immobilised platelets

Microscope slides were prepared and platelets adhered and fixed as outlined in the previous section. However, the slides were blocked with PBS containing 2% BSA (bovine serum albumin) for 30 minutes to block any non-specific binding sites. Anti-FITC conjugated P-selectin antibody (1:100) diluted in PBS containing 1% BSA was added after extensive washing steps with PBS for 1 hour and left in the dark. The slides were washed with PBS to ensure any unbound antibody was washed off and histomount with a coverslip was added.

2.4.3 Platelet adhesion under conditions of flow

Preliminary data performed with immobilised gAd under flow was performed in collaboration with Prof. R. Farndale and Dr N. Pugh at University of Cambridge. This adhesion assay was adapted from Siljander and colleagues (Siljander et al., 2004). In this adhesion assay labelled whole blood is flowed over immobilised protein such as collagen and platelet adhesion and thrombus formation can be observed under a confocal microscope. This assay will help us to understand if immobilised gAd can support platelet adhesion and thrombus formation under physiological conditions found in the veins and arteries. Blood from healthy donors was collected in 40 µM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) to inhibit prothrombin to thrombin cleavage and supplemented with 1 µM 3,3' dihexyloxacarbocyanine iodine ($DiOC_6$), a lipophilic fluorescent dye which binds to all cells and can be visualised under a microscope. Glass coverslips were coated with CRP-XL (10 µg/ml) or gAd (40 µg/ml) overnight at 4°C and then blocked by 1% BSA in HEPES buffer (36 mM NaCl, 2.7 mM KCl, 10 mM glucose, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 5 mM N-2-hydroxyethylpiperazine-N' -2-ethanesulfonic acid). The coverslip in a 125 µm deep flow chamber was mounted on to a FV300 laser-scanning confocal microscope (Olympus) and washed for 1 minute with HEPES buffer containing heparin 100 U/ml. Blood was drawn down to the chamber for 5 minutes using a syringe pump at 100, 300 and 1000s^{-1}. Platelet surface coverage and thrombus height was calculated using Image J software (NIH) as described by Pugh and colleagues (Pugh et al., 2010).

2.5 Methodologies for analysing proteins and signalling pathways in platelets

2.5.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Electrophoresis allows the separation of charged macromolecules in an electric field. When applied to a porous matrix such as a gel it can be used to separate molecules based on their size and charge. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), uses a combination of sodium dodecyl sulphate (SDS) and the polyacrylamide gels to separate proteins according to their molecular weight by electrophoretic migration. SDS-PAGE under denaturing conditions is a widely used method to separate proteins according to their size and not their charge (Towbin et al., 1979). Mercaptoethanol is used as a reducing agent to break disulfide bonds, returning the protein to its primary structure. SDS then gives the protein an overall negative charge [Figure 2.4].

SDS-PAGE is performed using a stacking gel which sits on top of the resolving gel and are composed of polyacrylamide. Polymerization of acrylamide is initiated by adding tetramethylethylenediamine (TEMED) and ammonium persulfate (APS). The TEMED acts as an electron carrier to activate the acrylamide monomer, providing an unpaired electron to convert the acrylamide monomer to a free radical. The activated monomer then reacts with an inactivated monomer to start the polymer chain reaction. The elongating polymer chains are randomly cross-linked by bisacrylamide, resulting in polymerization. When a current is applied, the negatively charged proteins will move towards the anode but due to the unique properties of the polyacrylamide gel i.e. pore size, smaller proteins will move faster compared to the larger proteins. As the stacking gel has a lower pH of 6.8 compared to the pH 8 of the resolving gel it allows the proteins to stack up and enter the resolving gel at the same time, thereby improving resolution. This is a discontinuous pH gel system that was originally described by Laemmli (Laemmli, 1970).

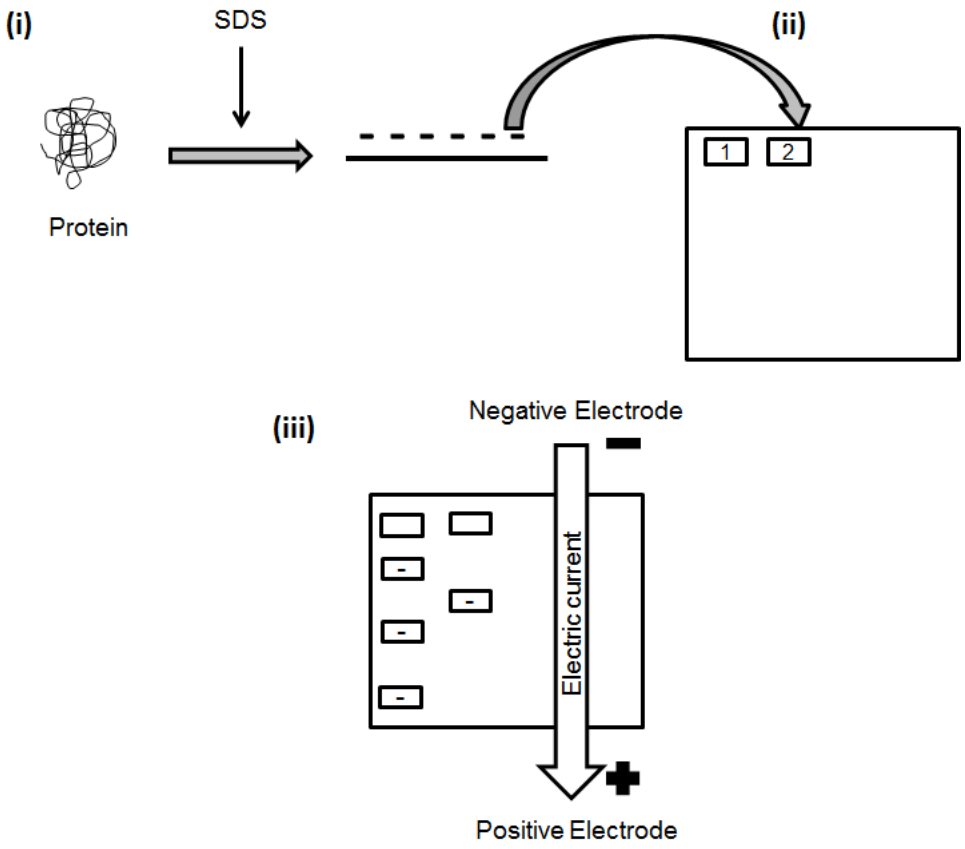


Figure 2.4: Overview of sodium dodecyl sulphate-polyacrylamide gel electrophoresis. (**i**) SDS binds to amino acid residues and gives uniform negative charge to the protein sample which is then further subjected to a cycle of heat induced denaturisation. (**ii**) Protein sample is loaded onto the SDS-PAGE gel. (**iii**) An electric current is applied to separate proteins according to size.

2.5.1.1 Preparation of platelet proteins for sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Washed platelets were prepared as described in section 2.2.1 and were pre-incubated with EGTA (1 mM) to promote non-aggregatory conditions by inhibiting integrin function (Gogstad et al., 1982). In some experiments, apyrase (2 U/ml) and indomethacin (10 μM) was added to eliminate platelet secondary mediated signalling by removing the β phosphate from ADP and inhibiting COX-1 and therefore TxA_2 synthesis respectively (Smith, 1971, Mustard et al., 1972). Platelets were incubated with various inhibitors under stirring conditions for 1 minute at 37°C and for longer incubation times, the platelets were initially stirred for 1 minute and left at 37°C under non-stirring conditions. The washed platelets were then stimulated under stirring conditions with an agonist for 90 seconds, unless otherwise stated. The reaction was stopped by the addition of an equal volume of 2x Laemmli buffer (0.5 M Tris HCL, 4% SDS, 20% glycerol and 10% mercaptoethanol) and stored at -20°C until required. The samples were boiled for 5 minutes before use to ensure denaturation of the proteins.

2.5.1.2 Measurement of protein concentrations

Protein concentration was measured using the Bio Rad DC Protein Assay Kit. The assay is a colorimetric reaction based on the Lowry method (Lowry et al., 1951), in which the reaction between the protein and an alkaline copper tartate solution leads to the reduction of the folin reagent and results in the development of a blue colour with maximal absorbance at 750 nm. BSA was used as a standard protein to compare protein concentration against the platelet protein sample. The BSA standards were prepared in the same buffer as the samples.

2.5.1.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis method

Protein lysates were separated by SDS-PAGE under denaturing conditions using a mini protean III gel kit (Bio Rad). A 3% stacking gel was used to allow all the proteins to stack up and enter the resolving gel at the same time. A 10% or 10-18% gradient gel was used as a resolving gel and placed in the electrophoresis tank containing running buffer (25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3). A total protein concentration of 20 µg unless otherwise stated was loaded into each well in the stacking gel and sample buffer was added to any free wells and was electrophoresed for 2.5 hours at 120 volts to ensure the proteins were fully distributed across the resolving gel.

2.5.2 Immunochemical techniques

2.5.2.1 Immunoblotting

Immunoblottting or western blotting is a sensitive and powerful method used to detect proteins of interest by using protein specific monoclonal and polyclonal antibodies. Once proteins have been separated by SDS-PAGE, they are transferred to an adhesive matrix or membrane such as nitrocellulose or polyvinylidene fluoride (PVDF) by application of an electrical current. The immobilised proteins on the membrane are allowed to interact with specific primary antibodies forming an antibody-antigen complex that can be identified by using different detection systems. The method of detection used in this study was an enhanced chemiluminescent (ECL) system. This method uses a species-specific secondary antibody, which is conjugated to an enzyme called horseradish peroxidase (HRP). Numerous secondary antibodies bind specifically to the primary antibody-antigen complex, acting to amplify the complex. The HRP catalyses the oxidation of chemiluminescent reagent, luminol, by hydrogen peroxide resulting in a luminescent signal which is detected by a photographic film.

2.5.2.2 Immunoblotting method

PVDF membranes were activated by pre-wetting with 10 ml of methanol for 1 minute, washed with 10 ml of water for 1 minute and left in 10 ml of transfer buffer until use (25 mM Tris base, 192 mM glycine, 20% (w/v) methanol, pH 8.3). Platelet protein samples were separated by SDS-PAGE as described in section 2.5.1.3 and the resolving gels was assembled into a transfer cassette [Figure 2.5]. The proteins were transferred into the activated PVDF membranes by applying 100 volts for 2.5 hours. After transfer membranes were blocked for 30 minutes with 10% BSA dissolved in Tris buffered saline-tween (TBS-T; 20 mM Tris, 137 mM NaCl, 0.1% tween-20; pH 7.6) or 5% milk protein dissolved in TBS-T to block any non-specific binding sites. Immunoblotting studies were performed using anti-phosphotyrosine 4G10 (1:1000), anti-Akt serine^{473} (1:2000), anti-β-tubulin (1:1000) and anti-PKC substrate (1:1000) dissolved in TBS-T containing 2% BSA and were left overnight at 4°C with gentle agitation. Membranes were then washed two times for 5 minutes with TBS-T to remove any unbound primary antibody and incubated either with a secondary antibody HRP-conjugated anti-mouse IgG (1:10000) or HRP-conjugated anti-rabbit (1:10000) as appropriate and HRP-conjugated anti-biotin (1:2000) for detection of biotinylated protein ladders for 1 hour at room temperature with gentle agitation. Membranes were subsequently washed five to six times for 15 minutes with TBS-T to remove any excess secondary antibody and membranes were developed with enhanced chemiluminescence solutions.

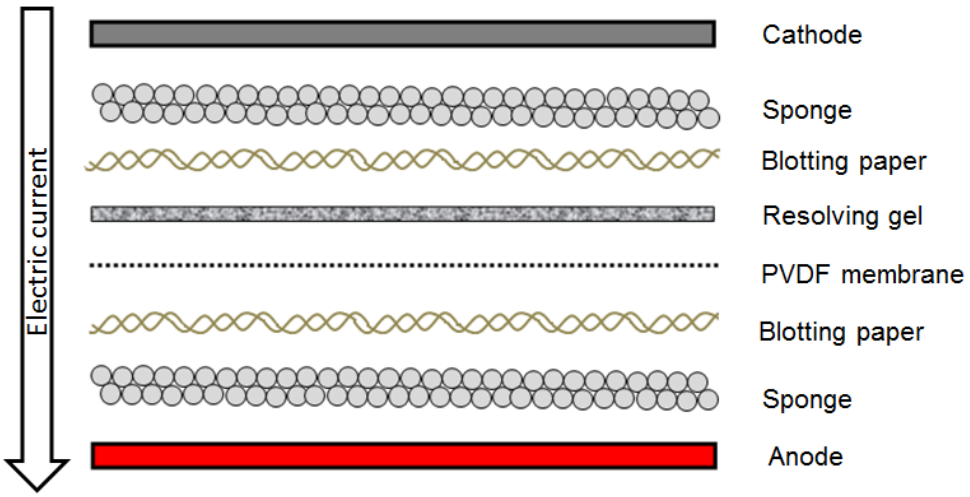


Figure 2.5: Assembly of western blotting cassette. The western blot cassette was assembled to allow proteins in the gel to be transferred to the PVDF membrane under an electric current.

2.5.2.3 Stripping and reprobing of membranes

Membranes can be stripped of antibodies and subsequently reprobed with different primary antibodies. In most cases this is performed to check for equal loading of proteins. Membranes were washed in stripping buffer composed of TBS-T, 5% mercaptoethanol and 2% SDS at 80°C for 20 minutes. Membranes were then washed three times with TBS-T for 10 minutes and the immunobloting procedure was repeated with the new primary antibody.

2.5.2.4 Densitometric measurement

Densitometric measurement of immunoblots was calculated using Image J software (NIH) and data was expressed as relative density to basal ± SEM or relative density/total ± SEM where appropriate. Statistical analysis was performed using the student *t* test. Briefly, a standard template box was drawn around the first band and was then cut and pasted onto the next band to maintain accuracy.

Chapter 3: The effects of adiponectin on platelet function

Adiponectin has been described as an endogenous anti-thrombotic factor but its direct effect on platelet function is not clear. Elbatarney and colleagues showed that fAd had no effect on platelet aggregation (Elbatarny et al., 2007). In contrast, Restituto and colleagues suggested that fAd can inhibit ADP and adrenaline-stimulated aggregation (Restituto et al., 2010). However, the effect of gAd on platelet aggregation remains to be determined. In this chapter we aim to investigate the effect of globular and full-length forms of adiponectin on platelet aggregation. In particular, look at the signalling events underlying any observed effects on aggregation. These experiments will allow us to determine the direct effect of adiponectin on platelet aggregation *in vitro*.

3.1 Aggregation and tyrosine phosphorylation studies with adiponectin

Initial experiments were designed to determine the effect of recombinant forms of adiponectin on platelet aggregation, since a recombinant form of the protein has been shown to stimulate platelet aggregation (Riba et al., 2008). Recombinant gAd (5-20 μg/ml) expressed from *E. Coli* was added to platelets and the effects on aggregation were monitored. Under these conditions, gAd stimulated platelet aggregation in a concentration-dependent manner, with a threshold response at 5 μg/ml and induced maximal aggregation at 20 μg/ml [Figure 3.1A]. In contrast, both collagen and CRP-XL induced maximal platelet aggregation at 10 μg/ml [Figure 3.1C and D]. Furthermore, we found platelet stimulation by gAd (20 μg/ml) also induced platelet dense-granule secretion as exemplified by the release of 0.38±0.07 nM ATP [Figure 3.1E]. However, this was significantly less when compared to other strong agonists such a GPVI specific agonist; CRP-XL or thrombin [Figure 3.1E]. These data suggest that only the globular form of adiponectin is able to stimulate full irreversible aggregation through platelet dense-granule secretion. In contrast, addition of fAd (20 μg/ml) to the platelet preparations failed to stimulate aggregation [Figure 3.1B].

These data suggested that different forms of adiponectin induced distinct effects on platelet aggregation with only gAd being pro-aggregatory.

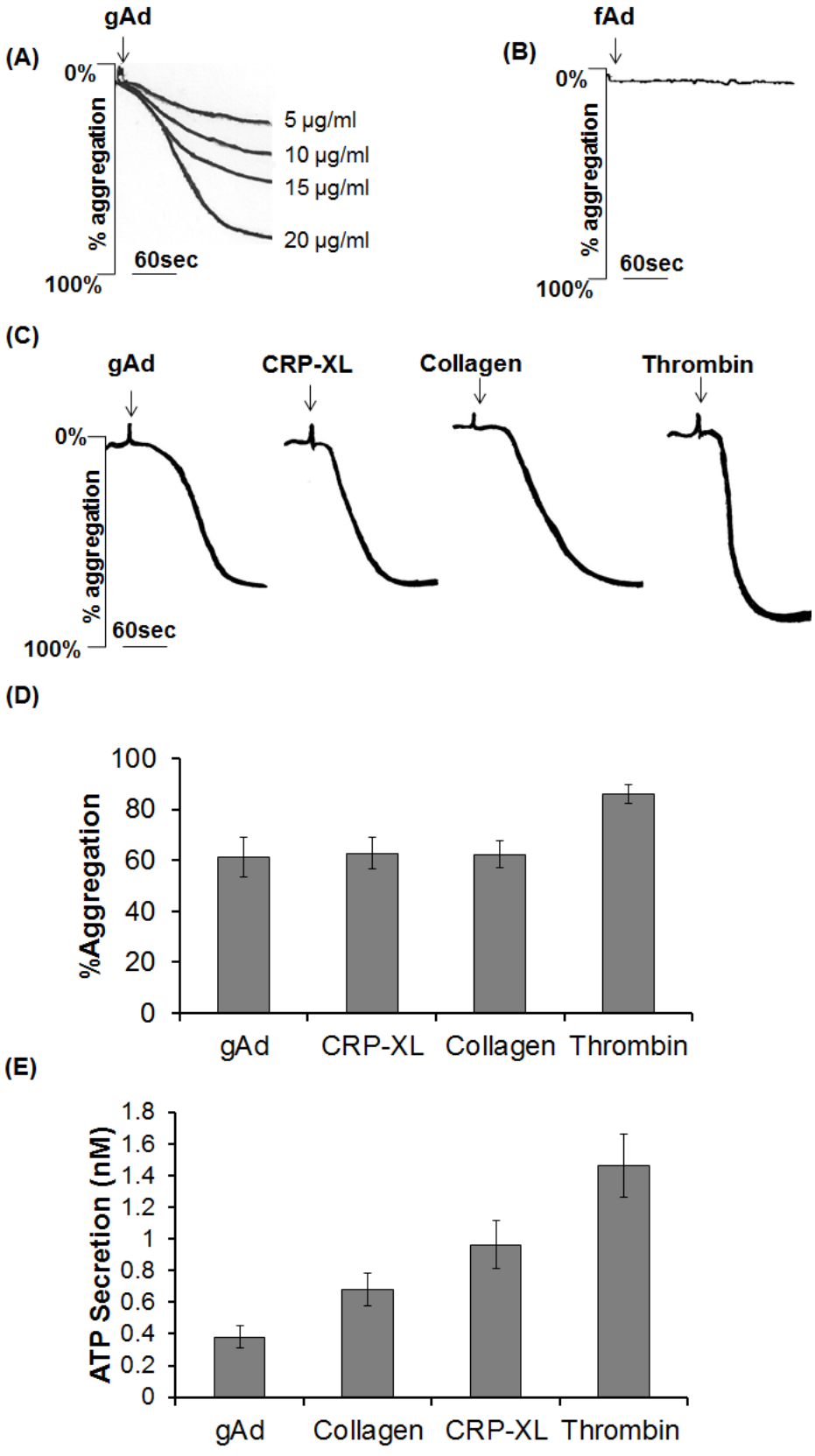


Figure 3.1: Globular adiponectin stimulates platelet aggregation. (**A**) Washed platelets ($2.5x10^8$ platelets/ml) were stimulated with a range of gAd concentrations (5-20 µg/ml) and the aggregation response was monitored for 4 minutes under constant stirring. (**B**) As in (A) except

platelets were stimulated with fAd (20 µg/ml). All representative aggregation traces are taken from three independent experiments performed with distinct blood donors. (**C**) and (**D**) Platelets were stimulated with gAd (20 µg/ml), collagen (10 µg/ml), CRP-XL (10 µg/ml) and thrombin (0.5 U/ml). The aggregation response was monitored for 4 minutes and expressed as %aggregation ± SEM of three independent experiments. (**E**) Washed platelets ($3x10^8$ platelets/ml) were stimulated with gAd (20 µg/ml), collagen (10 µg/ml), CRP-XL (10 µg/ml) and thrombin (0.5 U/ml). The luminescence response was monitored for 4 minutes under constant stirring and results were expressed as ATP nM secretion ± SEM of three independent experiments.

3.1.1 Characterisation of globular adiponectin-stimulated aggregation

Platelets are activated through surface receptors that enable the activation of integrin $\alpha_{IIb}\beta_3$ by inside-out signalling and thus allowing plasma fibrinogen to bind (Du et al., 1991). The fibrinogen acts as an inter-platelet bridge that facilitates platelet aggregation and forming the haemostatic plug. It was important to establish that responses observed with the platelet aggregometer were integrin-mediated aggregation and not platelet agglutination. To achieve this we used a number of pharmacological agents including Arginine-Glycine-Aspartate-Serine (RGDS), a peptide antagonist of the integrin $\alpha_{IIb}\beta_3$, and the extracellular Ca^{2+} chelator EGTA which prevents integrin function through removal of critical Ca^{2+} ions. GAd induced platelet aggregation was completely inhibited in the presence of RGDS (1 mM) ($p<0.05$), although platelet shape change still occurred as this is independent of integrin $\alpha_{IIb}\beta_3$ outside-in signalling [Figure 3.2A(i) and B]. Furthermore, incubation of washed platelets with EGTA (1 mM) totally ablated gAd-induced aggregation ($p<0.05$), thereby indicating that gAd-stimulated aggregation is integrin dependent [Figure 3.2A(ii) and B].

Platelet integrin $\alpha_{IIb}\beta_3$ activation requires signalling pathways that involve intracellular Ca^{2+} mobilisation, and numerous enzymes such as PLC, PKC and PI3K activation (Carpenter and Cantley, 1990, Atkinson et al., 2003, Lian et al., 2005, Harper and Poole, 2007). However, individual platelet agonists activate platelets by distinct mechanisms and therefore it was important to establish the importance of these key signalling events to platelet aggregation stimulated by gAd. Platelet aggregation was abolished by the intracellular Ca^{2+} chelator, 2-bis (2-aminophenoxy) ethane-N,N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM; 20 µM), consistent with the key role of Ca^{2+} in platelet aggregation [Figure 3.1A(iii) and B]. It is widely recognised that platelet activation can proceed through pathways that involve Src-kinases in response to both collagen and vWF (Nieswandt and Watson, 2003). A pan Src-kinase inhibitor, PP1 (20 µM) was able to fully ablate gAd-stimulated aggregation ($p<0.05$) [Figure

3.2A(iv) and B]. Similarly wortmannin (100 nM), a pan PI3K inhibitor, also completely abolished aggregation ($p<0.05$) [Figure 3.2A(v) and B]. In contrast, Ro 31-8220 (10 μM), a pan PKC inhibitor, only partially inhibited gAd-stimulated (20 μg/ml) aggregation from 73±3.28% to 37±5.45% ($p<0.05$), therefore indicating that platelet aggregation can proceed through pathways independent of PKC [Figure 3.2A(vi) and B]. Taken together, these data suggest that gAd-stimulated aggregation is tyrosine kinase-dependent mechanism that requires PI3K and to a lesser degree an isoform of PKC.

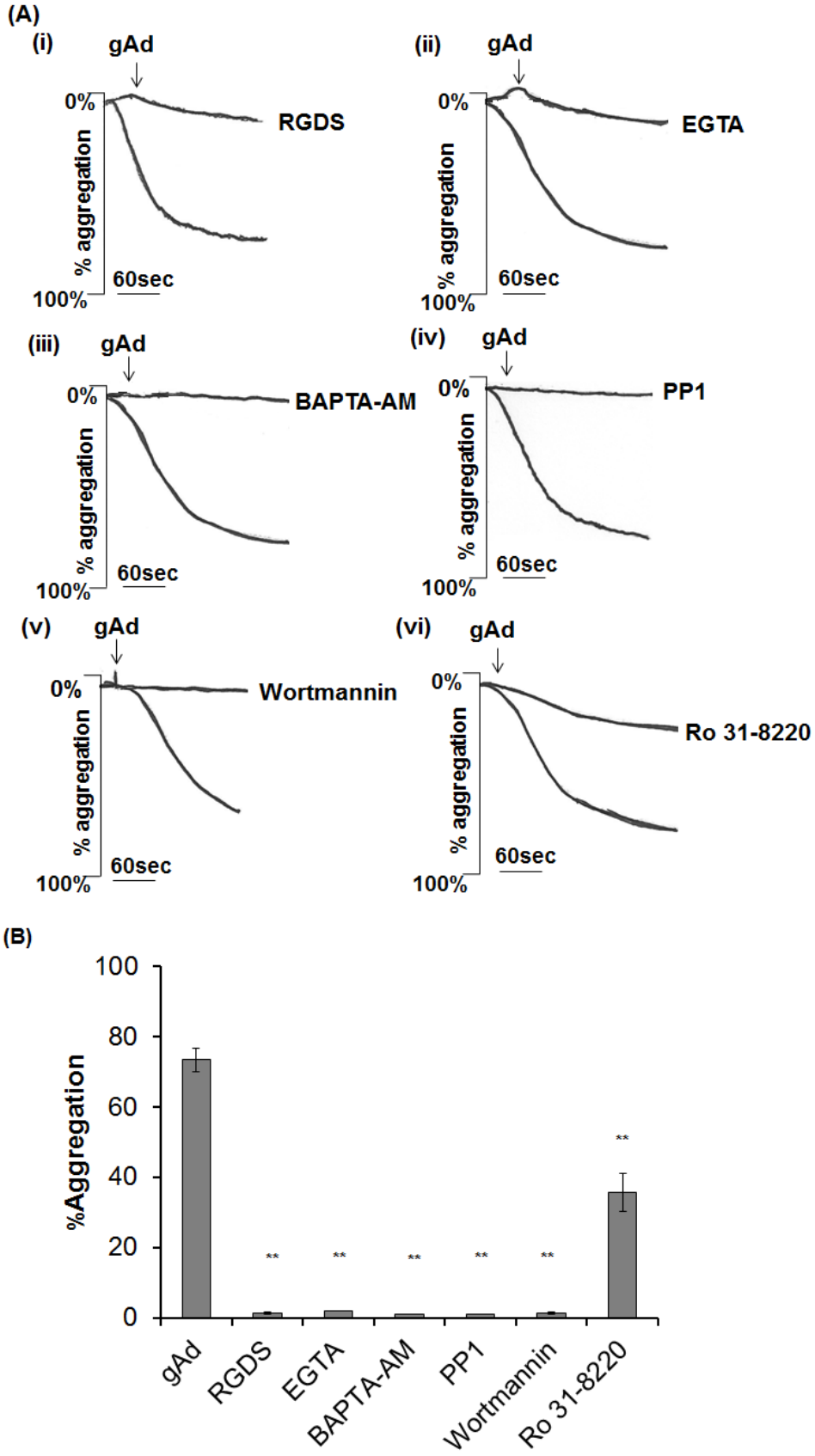


Figure 3.2: Characterisation of globular adiponectin-stimulated aggregation. (**A**) Washed platelets (2.5x10^8 platelets/ml) were stimulated

with gAd (20 µg/ml) in the absence and presence of RGDS (1 mM), EGTA (1 mM), BAPTA-AM (10 µM), PP1 (20 µM), wortmannin (100 nM) and Ro 31-8220 (10 µM) and the aggregation response was monitored for 4 minutes under constant stirring. All representative aggregation traces are taken from four independent experiments performed with distinct blood donors. (**B**) Representative of data in (A) and results are shown as the mean ± SEM of four independent experiments, where ** indicates $p<0.005$ compared to gAd alone with the inhibitors.

3.1.2 Globular adiponectin stimulates tyrosine phosphorylation

These initial platelet aggregation experiments with gAd revealed an absolute requirement of Src-kinase isoforms in gAd-stimulated aggregation [Figure 3.2]. Since activation of Src-kinase isoforms leads to the phosphorylation of platelet proteins on tyrosine residues (Thomas and Brugge, 1997, Ezumi et al., 1998), the role of Src-kinases in gAd-stimulated platelets was investigated. In the first instance the ability of gAd to induce tyrosine phosphorylation was investigated. GAd (20 μg/ml) induced a robust increase in tyrosine phosphorylation of platelet proteins in whole cell lysates, with prominent bands at 38, 45, 70 and 140 kDa [Figure 3.3]. However, proteins at 60 kDa region were found to be basally phosphorylated and maintained their phosphorylation state after stimulation with gAd. Platelets stimulated with gAd in the presence of PP1 (20 μM), the pan Src family kinase inhibitor, completely inhibited tyrosine phosphorylation of these platelet proteins, including proteins at 70 and 140 kDa, previously seen to be stimulated by gAd [Figure 3.3B]. Importantly, PP3 (20 μM), the inactive structural analogue of PP1, had no effect on tyrosine phosphorylation [Figure 3.3]. Taken together, these data suggest that gAd activate platelets through a tyrosine kinase-dependent mechanism mediated at least in part by Src-kinases. Importantly, these tyrosine phosphorylation experiments were performed in the presence of EGTA, indomethacin and apyrase which remove the positive feedback of soluble platelet agonists such as TxA_2 and ADP respectively.

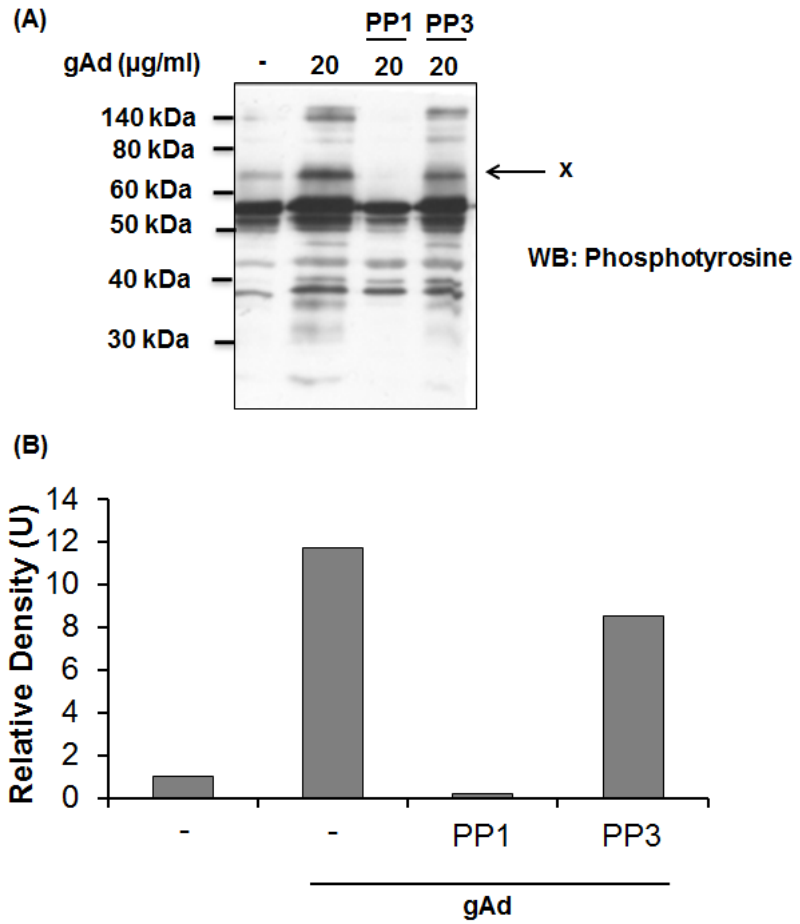


Figure 3.3: Globular adiponectin stimulates tyrosine phosphorylation of platelet proteins. (**A**) Washed platelets ($5x10^8$ platelets/ml) were incubated with apyrase (2 U/ml), indomethacin (10 µM) and EGTA (1 mM) for 20 minutes and then stimulated with gAd (20 µg/ml) in the presence of either PP1 (20 µM) or PP3 (20 µM) for 90 seconds. Proteins were resolved on 10-18% gradient polyacylamide gel SDS-PAGE and immunoblotted using anti-phosphotyrosine antibody. (**B**) Densitometric measurement of phosphotyrosine at band labelled "x" and expressed as relative density to basal. (This confirms previous data from Riba et al., 2007)

3.2 The role of secondary mediators in globular adiponectin-stimulated aggregation

Platelets release secondary mediators such as ADP and TxA_2 that reinforce platelet activation and thrombus formation. Since early platelet aggregation experiments with gAd [Figure 3.1] had established that the release of dense-granules was required for maximal aggregation, the role of ADP and TxA_2 was investigated in this process. To achieve this platelet aggregation was monitored in the presence of ADP receptor antagonists, A3P5P and MRS2395 to inhibit the potential effects of ADP signalling through $P2Y_1$ and $P2Y_{12}$ receptors respectively (Atkinson et al., 2001, Kunapuli et al., 2003), or indomethacin to prevent TxA_2 formation (Vane, 1971, Charo et al., 1977). It was important to ensure that the concentration of these inhibitors was able to inhibit these pathways.

In the first instance the effectiveness of the ADP receptor antagonists were tested against ADP-induced aggregation to confirm their effectiveness. The addition of A3P5P (300 µM) and MRS2395 (10 µM) (Atkinson et al., 2001) was sufficient to ablate ADP-mediated aggregation when combined, suggesting the concentration of these antagonists was sufficient to prevent ADP receptor signalling [Figure 3.4]. Studies have shown that indomethacin can irreversibly inhibit TxA_2 synthesis at 10 µM through the inhibition of COX-1 (Maderna et al., 1985).

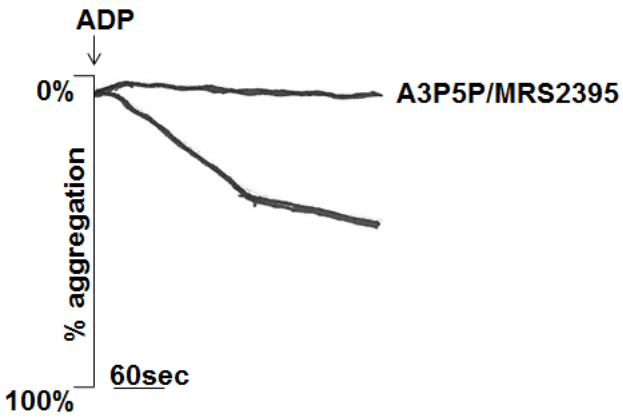


Figure 3.4: ADP receptor antagonists inhibit ADP-mediated aggregation. Washed platelets ($2.5x10^8$ platelets/ml) supplemented with fibrinogen (20 µg/ml) were stimulated with ADP (50 µM) and the aggregation response was monitored for 4 minutes under constant stirring in the presence or absence of ADP receptor antagonists; A3P5P (300 µM) and MRS2395 (10 µM).

3.2.1 Globular adiponectin-stimulated aggregation requires secondary platelet agonists

In the first instance the role of ADP and TxA_2 on gAd-stimulated aggregation was investigated. Since gAd stimulates platelets through a tyrosine kinase-dependent mechanism [Figure 3.3], the effects of gAd was compared with collagen. Stimulation of platelets with gAd (20 μg/ml) induced full irreversible aggregation of 68±0.60% (EC_{50} 17.26 μg/ml), which was reduced to 58±1.5% ($p<0.05$) by the presence of A3P5P/MRS2395 (EC_{50} 29.61 μg/ml), to 47±0.90% ($p<0.05$) by indomethacin (EC_{50} 29.03 μg/ml) and then further reduced to 33±1.90% ($p<0.05$) when all three inhibitors were combined (EC_{50} 27.83 μg/ml) [Figure 3.5A and C].

In contrast, at threshold concentrations of gAd (10 μg/ml) the inhibitors had potent inhibitory effect on aggregation [Figure 3.5B]. The ADP receptors antagonists alone reduced aggregation from 25±3.93% to 7±3.17%, and indomethacin reduced aggregation to 6±1.20%, while in combination the inhibitors ablated aggregation. As can be seen in Figure 3.5C, the presence of the inhibitors led to a rightward shift in the concentration response curve for gAd.

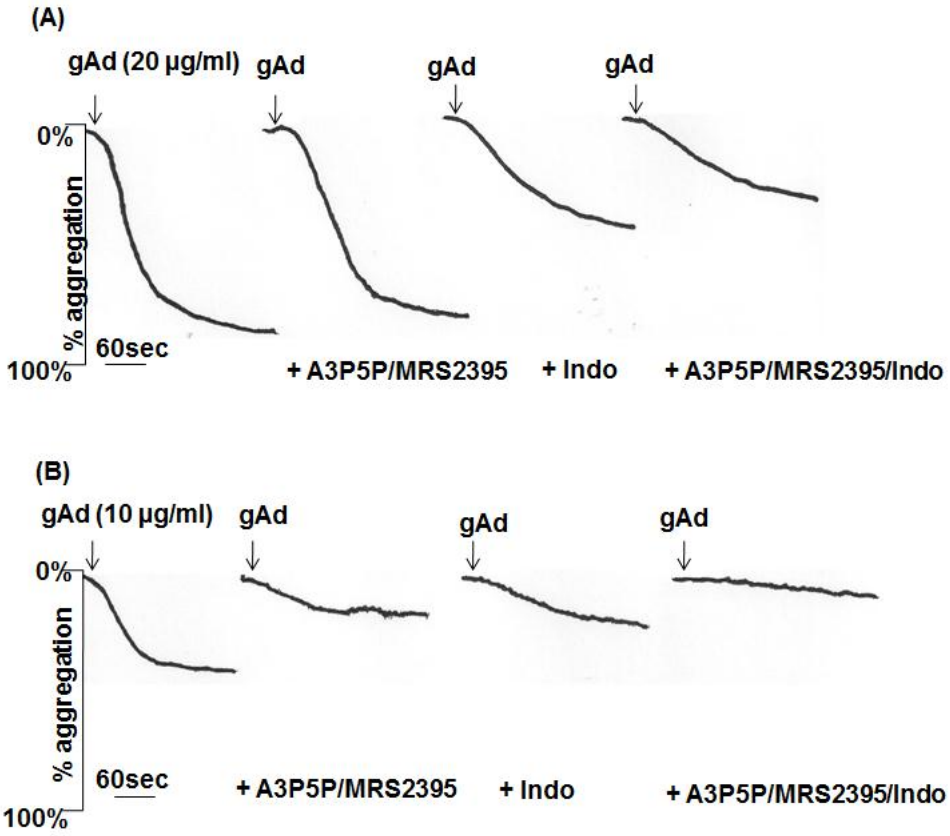


Figure 3.5: Globular adiponectin-stimulated aggregation is dependent on secondary platelet mediators. (**A**) Washed platelets ($2.5x10^8$ platelets/ml) were stimulated with gAd (20 µg/ml) in the presence or absence of ADP receptor antagonists; A3P5P (300 µM), MRS2395 (10 µM) or cyclo-oxygenase inhibitor indomethacin (10 µM). (**B**) As in (A) except platelets were stimulated with gAd (10 µg/ml). All representative aggregation traces are taken from four independent experiments performed with distinct blood donors.

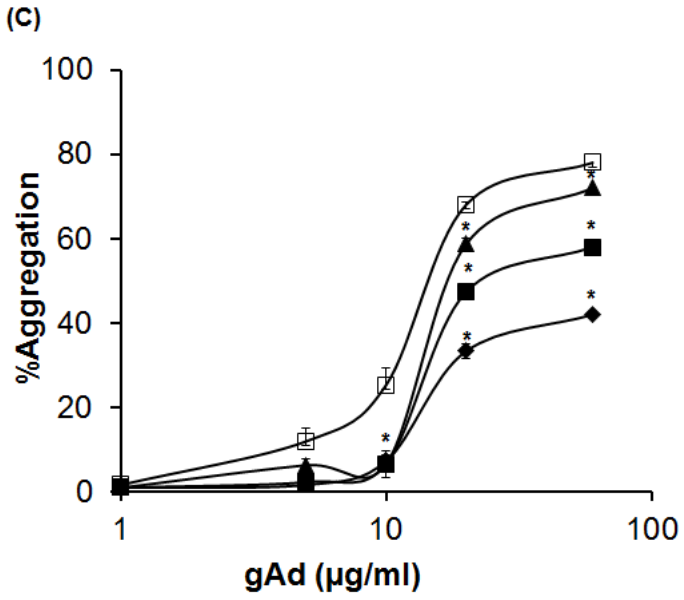


Figure 3.5: Globular adiponectin-stimulated aggregation is dependent on secondary platelet mediators. (**C**) Washed platelets were stimulated with gAd dose response (1-50 µg/ml) alone (⊟), in the presence of A3P5P (300 µM) and MRS2395 (10 µM) (▲), in the presence of cyclo-oxygenase inhibitor indomethacin (10 µM) (■) or in the presence of A3P5P, MRS2395 and indomethacin combined (◆). Results are shown as the mean ± SEM of four independent experiments, where * indicates $p<0.05$ compared with gAd alone to the inhibitors.

Under these identical experimental conditions, the role of ADP and TxA_2 on collagen-stimulated aggregation was also investigated. Stimulation of platelets with collagen (10 µg/ml) induced full irreversible aggregation of 80±3.97% (EC_{50} 3.43 µg/ml). This was reduced to 72±2.83% by the presence of the ADP receptor antagonists (EC_{50} 4.04 µg/ml), to 36±4.88% by the presence of indomethacin (EC_{50} 10.18 µg/ml) and then further reduced to 22±2.01% ($p<0.05$) when all three inhibitors were combined (EC_{50} 17.88 µg/ml) [Figure 3.6A and C]. In contrast, platelets stimulated with threshold concentration of collagen (0.25 µg/ml) also showed a strong dependency on secondary mediators as evidenced by the rightward shift in the concentration response curve for collagen [Figure 3.6B and C]. Thus, gAd stimulates platelet aggregation through pathways that are both dependent and independent of secondary mediators. These data also demonstrate that gAd behaves in a manner similar to collagen in that secondary mediators are required to induce a maximal aggregation response (Dawood et al., 2007).

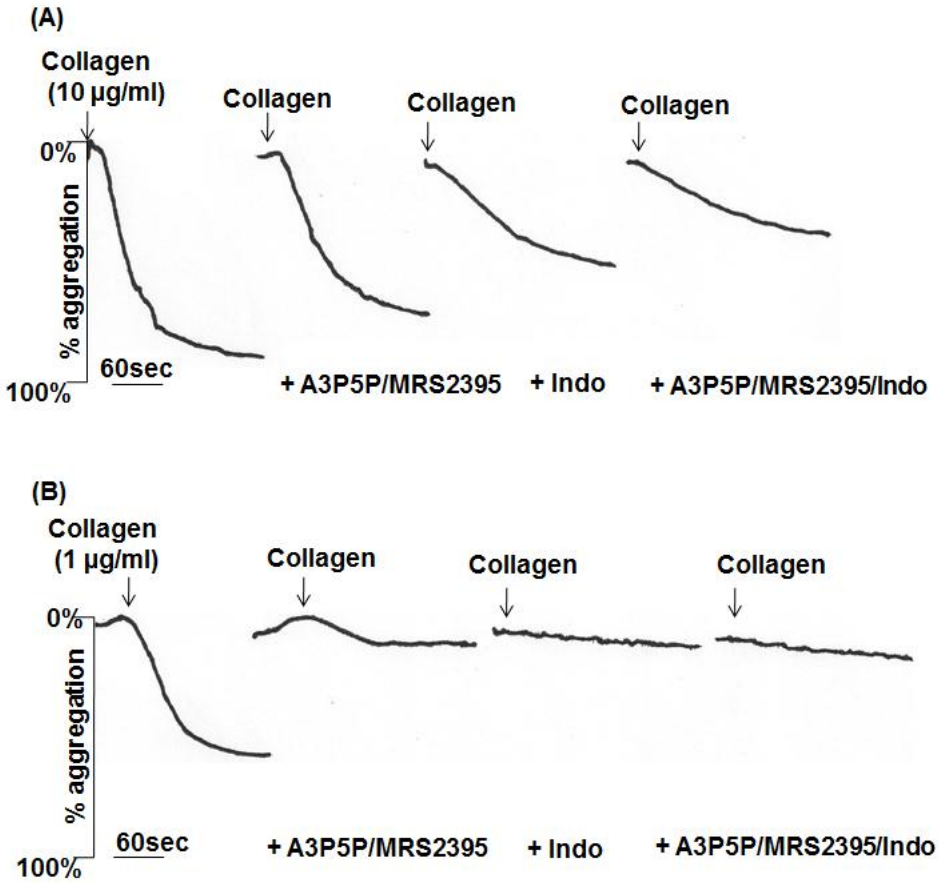


Figure 3.6: Collagen-stimulated aggregation is dependent on secondary platelet mediators. (**A**) Washed platelets ($2.5x10^8$ platelets/ml) were stimulated with collagen (10 µg/ml) in the presence or absence of ADP receptor antagonists; A3P5P (300 µM), MRS2395 (10 µM) or cyclo-oxygenase inhibitor indomethacin (10 µM). (**B**) As in (A) except platelets were stimulated with collagen (1 µg/ml). All representative aggregation traces are taken from four independent experiments performed with distinct blood donors.

(C)

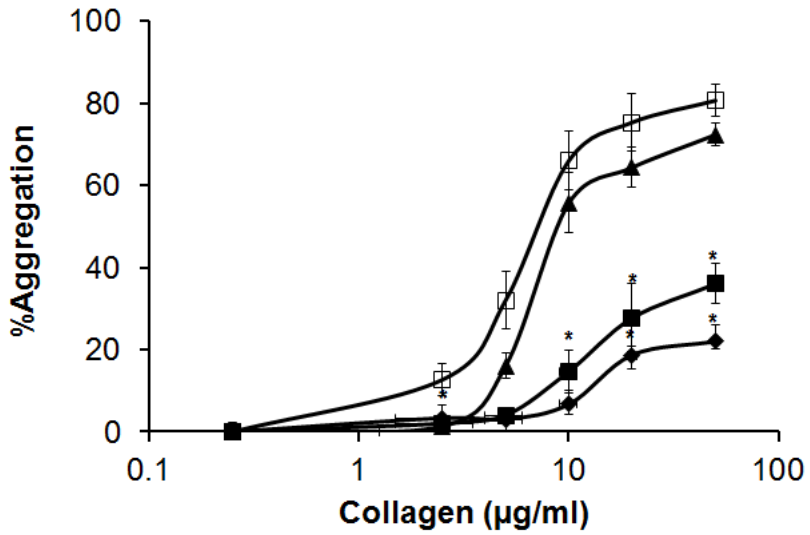


Figure 3.6: Collagen-stimulated aggregation is dependent on secondary platelet mediators. (**C**) Platelets were stimulated with collagen dose response (0.25-50 µg/ml) alone (⊟), in the presence of A3P5P (300 µM) and MRS2395 (10 µM) (▲), in the presence of cyclo-oxygenase inhibitor indomethacin (10 µM) (■) or in the presence of A3P5P, MRS2395 and indomethacin combined (◆). Results are shown as the mean ± SEM of four independent experiments, where * indicates $p<0.05$ compared with collagen alone to the inhibitors.

3.2.2 Globular adiponectin can synergise with G_i-coupled platelet agonists

Since gAd-induced platelet aggregation required secondary mediators to induce a maximal response, this led to the possibility that these agonists could synergise with the adipokine to increase aggregation. Platelets were stimulated with gAd (5 μg/ml) which induced an aggregation response of 16±2.1%. The presence of ADP (1 μM) potentiated gAd (5 μg/ml)-stimulated aggregation from 12±4.3% to 63±2.7% ($p<0.05$) [Figure 3.7A]. Having established that synergism could take place, the identification of the ADP receptor responsible was investigated. This synergistic response was maintained in the presence of A3P5P, but reduced in the presence of MRS2395 [Figure 3.7B].

We next examined the potential for adrenaline and TxA_2 to synergise with gAd. Similar to ADP, adrenaline (10 μM) synergised with gAd to increase threshold platelet aggregation response from 18±0.7% to 32±2.7% ($p<0.05$) [Figure 3.8A], without inducing aggregation itself. In contrast, the TxA_2 mimetic U46619 failed to synergise with gAd at any concentration tested whilst inducing full aggregation by itself at 10 μM [Figure 3.83]. These data suggest a role of $P2Y_{12}$ G_i and G_z-coupled G-proteins in the potentiation of gAd-stimulated aggregation.

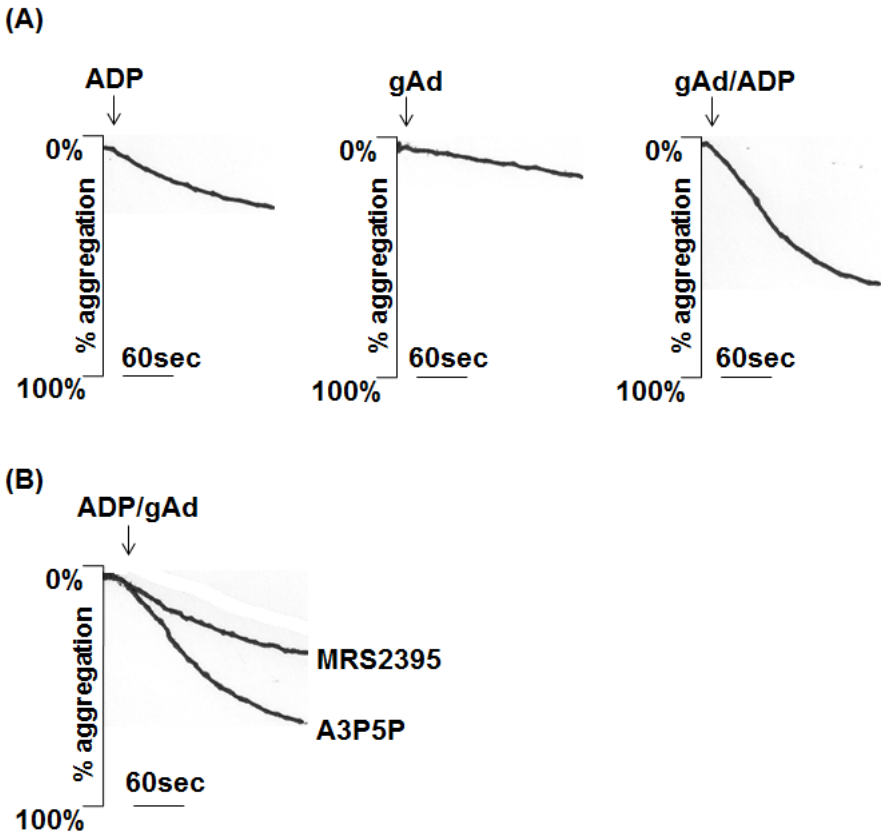
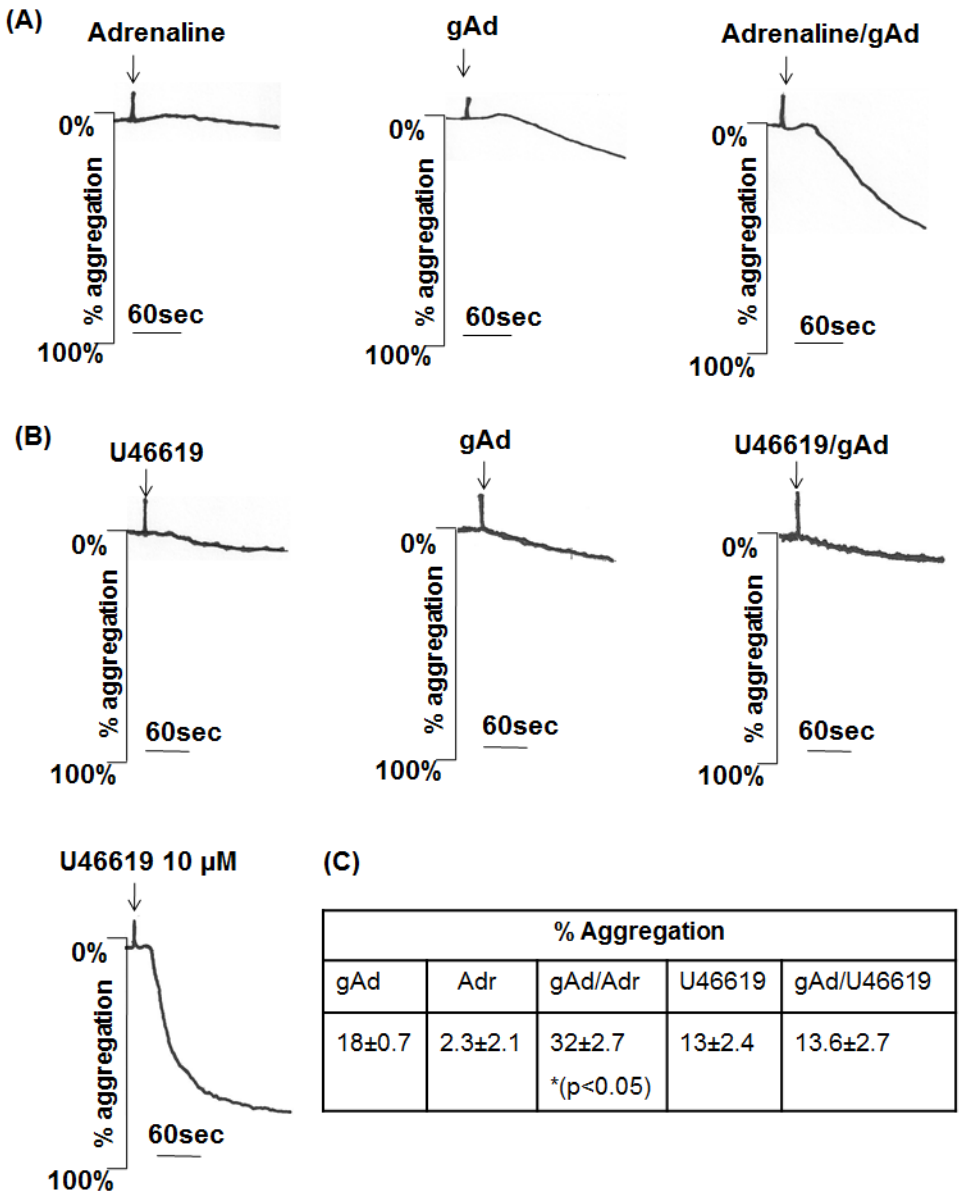


Figure 3.7: Globular adiponectin synergies with G_i-coupled agonists to promote platelet aggregation. (**A**) Washed platelets ($2.5x10^8$ platelets/ml) were stimulated with sub-threshold concentrations of gAd (5 µg/ml) alone, ADP (1 µM) alone or gAd and ADP combined and the aggregation response was monitored for 4 minutes under constant stirring. (**B**) Platelets were stimulated with both gAd (5 µg/ml) and ADP (1 µM) in the presence or absence of ADP receptor antagonists A3P5P (300 µM) or MRS2395 (10 µM). All traces are representative of data obtained from four independent experiments.



% Aggregation				
gAd	Adr	gAd/Adr	U46619	gAd/U46619
18±0.7	2.3±2.1	32±2.7 *(p<0.05)	13±2.4	13.6±2.7

Figure 3.8: Globular adiponectin synergies with G_i-coupled agonists to promote platelet aggregation. (**A**) Washed platelets (2.5x10^8 platelets/ml) were stimulated with sub-threshold concentrations of gAd (5 µg/ml) alone or adrenaline (10 µM) or combined. (**B**) As in (A) except platelets were stimulated with gAd (5 µg/ml) and the TxA_2 mimetic U46619 (0.5 µM). All traces are representative of data obtained from four independent experiments. (**C**) Quantified data of (**A**) and (**B**), where * indicates p<0.05 compared with gAd in the presence and absence of adrenaline.

3.2.3 The influence of thromboxane signalling on platelet dense-granule secretion in globular adiponectin-stimulated aggregation

To further clarify the role of TxA_2 and ADP on gAd-stimulated aggregation, platelet dense-granule secretion was examined with lumi-aggregation in the presence and absence of indomethacin and ADP receptor antagonists. Stimulation of platelets with gAd (20 μg/ml) induced dense-granule secretion as exemplified by the release of 0.44±0.08 nM ATP [Figure 3.9]. However, ATP secretion stimulated by gAd was unaffected by the presence of the ADP receptor antagonists (0.42±0.06 nM ATP secretion), but was abolished under conditions of TxA_2 blockade by indomethacin ($p<0.05$), suggesting that secretion was driven by TxA_2.

To explore this further we used the TxA_2 mimetic U46619 (Parise et al., 1982, Zatta et al., 1993). The inhibition of secretion in the presence of indomethacin was restored to 0.25±0.065 nM ATP by the addition of U46619 (5 μM) ($p<0.05$) when compared to indomethacin alone while the effects of U46619 were abolished by the thromboxane TPα/β receptor antagonist SQ29548 (10 μM) ($p<0.05$) [Figure 3.9] and the pan PKC inhibitor Ro 31-8220. Taken together, these data may suggest that gAd induced secretion requires the synthesis of TxA_2, which drives PKC-dependent secretion of ADP from dense-granules to further amplify gAd-stimulated aggregation.

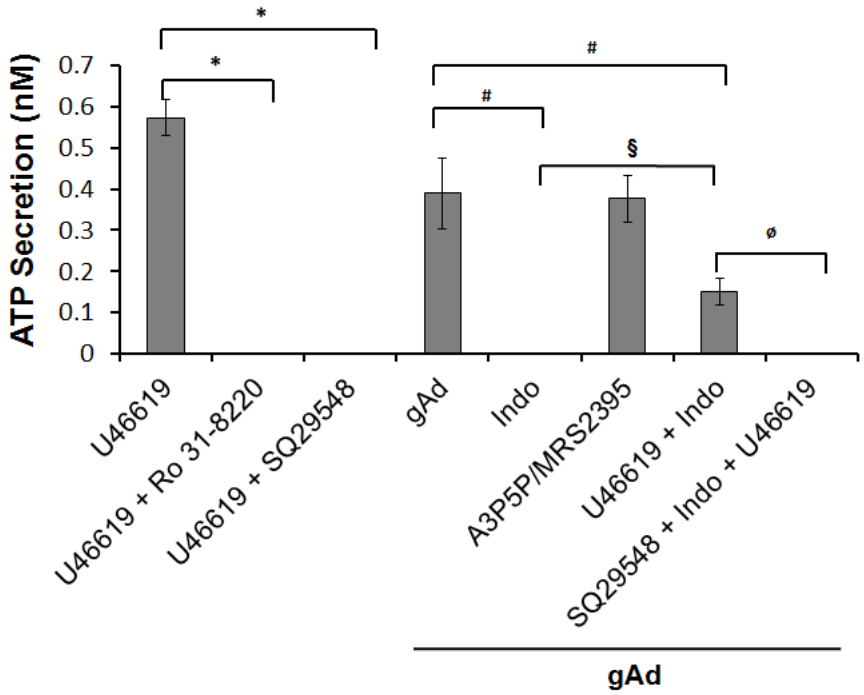


Figure 3.9: Thromboxane drives platelet dense-granule secretion in response to globular adiponectin. Washed platelets ($3x10^8$ platelets/ml) were stimulated with gAd (20 µg/ml) in the presence and absence of indomethacin (10 µM), SQ29548 (10 µM) A3P5P (300 µM), MRS2395 (10 µM) and U46619 (5 µM) and the luminescence response was monitored for 4 minutes under constant stirring. Results were expressed as ATP nM secretion ± SEM of four experiments, where #, § and ø indicates $p<0.05$ compared with gAd stimulation in the presence of the inhibitors and * indicates $p<0.05$ compared with U46619 stimulation in the presence of the inhibitors.

3.2.4 The influence of platelet derived mediators on tyrosine phosphorylation stimulated by globular adiponectin

Since gAd-mediated aggregation required both Src-family kinases and the release of ADP and TxA_2, the potential relationship between these two events was investigated. Hence, the influence of platelet derived TxA_2 and ADP on gAd-induced tyrosine phosphorylation was examined. Consistent with earlier experiments [Figure 3.3], gAd (20 µg/ml) stimulated a marked increase in tyrosine phosphorylation, with the most prominent bands observed at 45, 50, 72, and 140 kDa [Figure 3.10]. After 90 seconds, at which point gAd induced tyrosine phosphorylation was maximal and reproducible (Riba et al., 2008), the inhibitors of $P2Y_1$ and $P2Y_{12}$ inhibitors and indomethacin were tested. A3P5P and MRS2395, either alone or in combination, failed to influence phosphorylation induced by gAd [Figure 3.10]. Consistent with this data the presence of indomethacin did not influence tyrosine phosphorylation induced by the adipokine [Figure 3.10]. Thus, tyrosine phosphorylation in response to gAd is independent of secondary mediators.

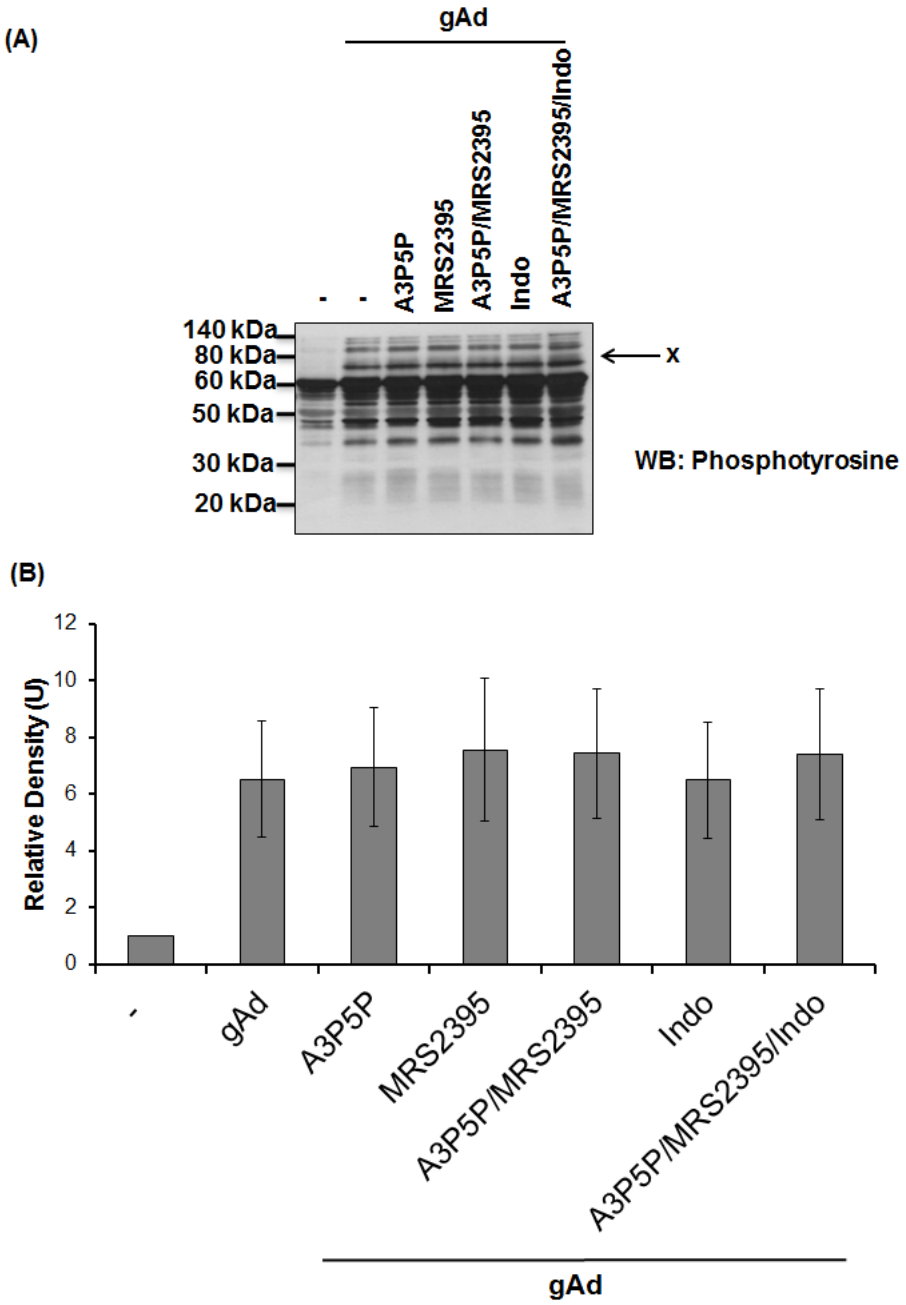


Figure 3.10: Secondary mediators do not influence globular adiponectin-stimulated tyrosine phosphorylation. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with gAd (20 µg/ml) in the presence or absence of ADP receptor antagonists; A3P5P (300 µM), MRS2395 (10 µM) and cyclo-oxygenase inhibitor indomethacin (10 µM). Platelets were lysed, separated by SDS-PAGE and blotted for phosphotyrosine containing proteins. (**B**) Densitometric measurement of phosphotyrosine at band labelled "x" and expressed as relative density to basal ± SEM of three independent experiments.

As a comparison the importance of secondary mediators to tyrosine phosphorylation in other tyrosine kinase agonists such as collagen was tested. Stimulation of washed platelets by collagen (10 µg/ml) induced tyrosine phosphorylation of a number of platelet proteins, with prominent bands at 28, 45, 72, 100 and 120 kDa [Figure 3.11]. Similar to gAd-induced tyrosine phosphorylation, proteins at 60 kDa were found to be basally phosphorylated and maintained their phosphorylation state after stimulation. Consistent with the previous data obtained from gAd and previously published work with collagen (Atkinson et al., 2003), the ADP receptor antagonists, indomethacin or in combination had no influence on tyrosine phosphorylation [Figure 3.11]. These data are consistent with a mechanism whereby tyrosine phosphorylation is independent of platelet derived secondary mediators.

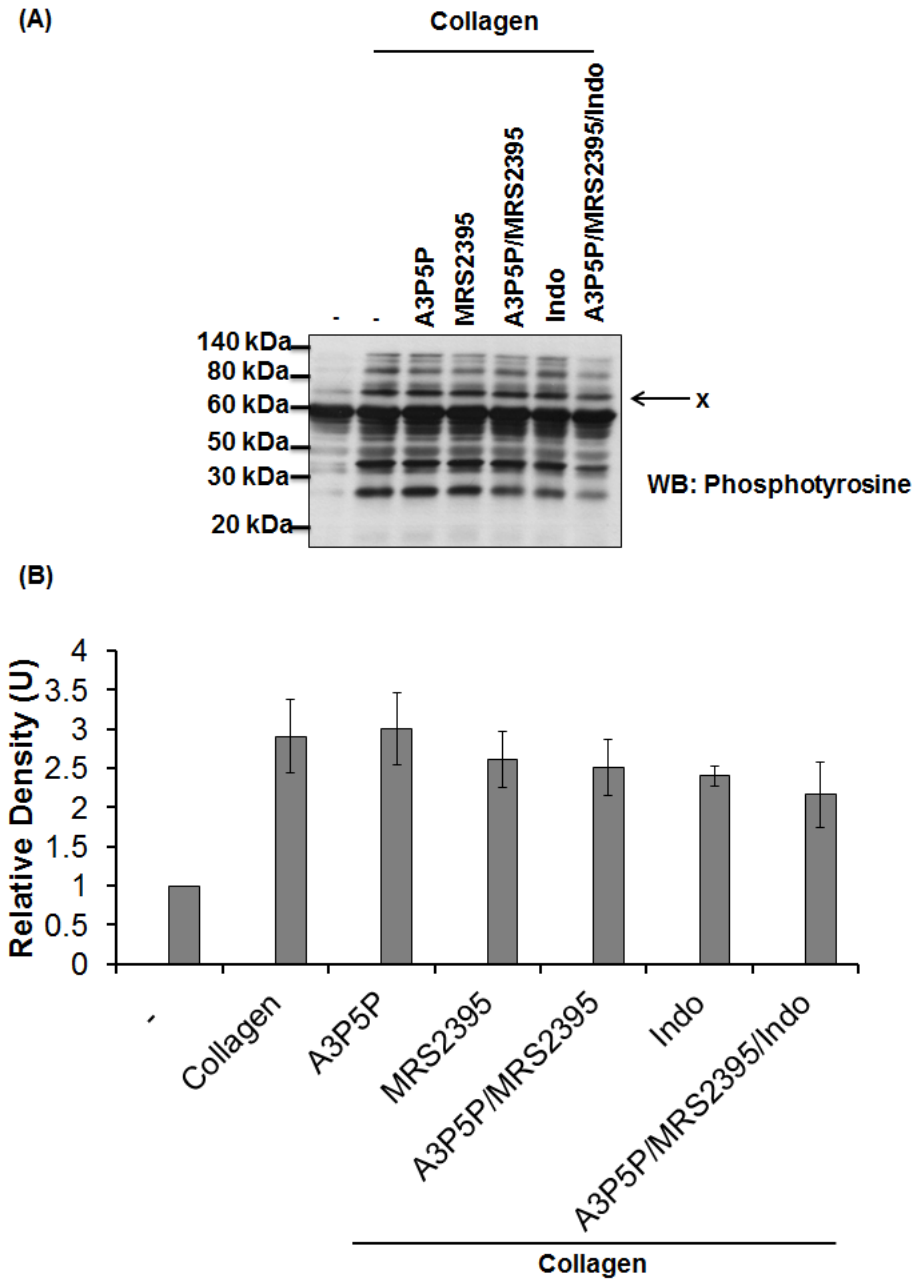


Figure 3.11: Secondary mediators do not influence collagen-stimulated tyrosine phosphorylation. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with collagen (10 µg/ml) in the presence or absence of ADP receptor antagonists; A3P5P (300 µM), MRS2395 (10 µM) and cyclo-oxygenase inhibitor indomethacin (10 µM). Platelets were lysed, separated by SDS-PAGE and blotted for phosphotyrosine containing proteins. (**B**) Densitometric measurement of phosphotyrosine at band labelled "x" and expressed as relative density to basal ± SEM of three independent experiments.

3.2.5 The direct effect of secondary mediators on tyrosine phosphorylation

To confirm that platelet derived secondary mediators do not influence tyrosine phosphorylation in gAd-stimulated platelets, the ability of ADP and TxA_2 to induce tyrosine phosphorylation by itself was investigated. Washed platelets in non-aggregatory conditions were stimulated for 90 seconds with ADP (10 µM) and no increase in tyrosine phosphorylation was detected [Figure 3.12]. The G_q-coupled agonist, U46619 (10 µM)-a thromboxane mimetic and G_z-coupled agonist adrenaline also did not stimulate increase in tyrosine phosphorylation levels [Figure 3.12]. These data suggest that platelet derived ADP and TxA_2 do not play a significant role in gAd-stimulated tyrosine phosphorylation.

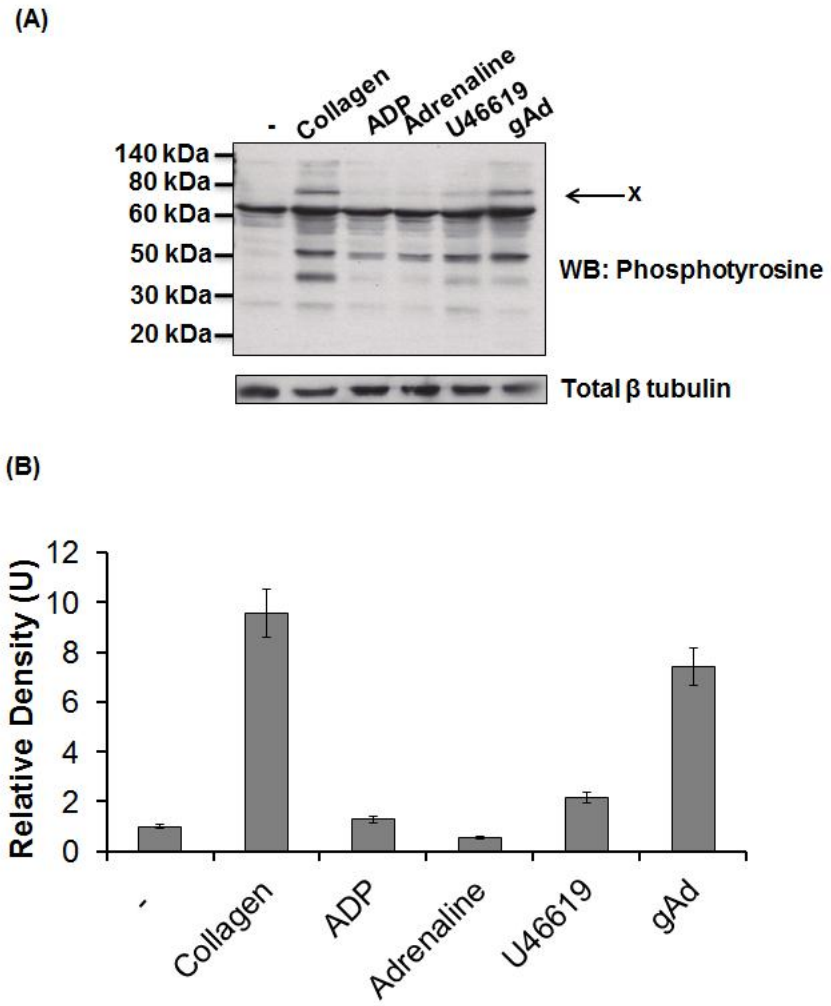


Figure 3.12: Platelet secondary agonists do not induce tyrosine phosphorylation. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated for 90 seconds with collagen (10 µg/ml), ADP (10 µM), adrenaline (10 µM), thromboxane mimetic U46619 (10 µM) and gAd (20 µg/ml). Platelets were lysed, separated by SDS-PAGE and blotted for phosphotyrosine containing proteins, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphotyrosine at band labelled "x" and expressed as relative density to basal ± SEM of three independent experiments.

3.3 Role of PKC in globular adiponectin-stimulated platelet aggregation

PKC is a family of serine-threonine kinases that are known to play a key role in platelet secretion (Harper and Poole, 2007). Since early data indicate that secretion of ADP was required for gAd-stimulated platelet aggregation, the role of PKC was examined in this process using the general PKC inhibitor, Ro 31-8220 (10 μM). Under these conditions, PKC blockade abolished aggregation induced by low concentrations of gAd (1-15 μg/ml) [Figure 3.13], whereas gAd-induced (20 μg/ml) platelet aggregation was inhibited by 53±3.8% ($p<0.05$). These data would suggest that PKC is required for maximal aggregation induced by gAd.

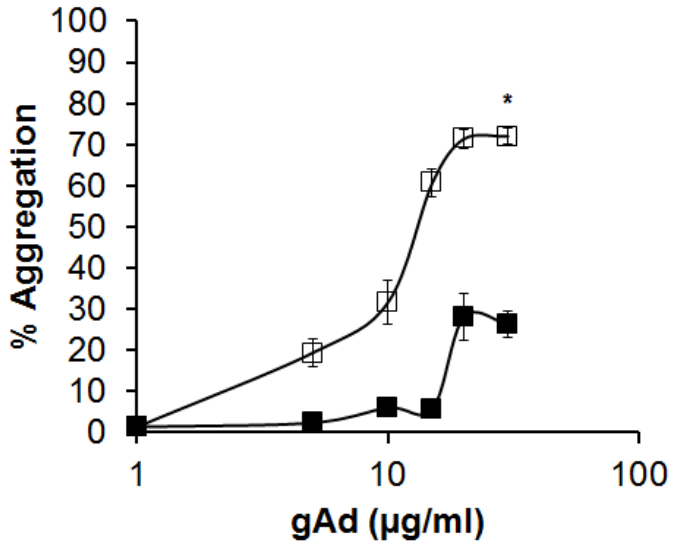


Figure 3.13: Globular adiponectin-stimulated platelet aggregation occurs through PKC-dependent and independent pathways. (**A**) Washed platelets ($2.5x10^8$ platelets/ml) were stimulated with gAd (1-50 µg/ml) and the aggregation response was monitored for 4 minutes under constant stirring in the absence (⊟) or presence of Ro 31-8220 (10 µM) (■). Results are shown as the mean ± SEM of four independent experiments, where * indicates $p<0.05$ compared to gAd alone with Ro 31-8220.

3.3.1 PKC substrate phosphorylation by globular adiponectin

To confirm the activation of PKC by gAd, and the ability of Ro 31-8220 to inhibit PKC activation, an antibody that recognises phosphorylation substrate by PKC isoforms (sequence (R/K)X(S*)(Hyd)(R/K)) was used (Pearson and Kemp, 1991, Nishikawa et al., 1997). GAd increased phosphorylation of a number of PKC substrates with molecular weights of 60, 80, 100 and 140 kDa in a concentration-dependent manner [Figure 3.14]. Although the ability to activate PKC was modest in comparison to phorbol 12-myristate 13-acetate (PMA) (300 nM) [Figure 3.14 and 3.15], densitometric measurements of these bands showed different PKC substrate phosphorylation kinetics. For example, PKC substrate phosphorylation levels at 140 kDa were found to be maximal between gAd 5-10 μg/ml stimulation [Figure 3.14B]. In contrast, PKC substrate phosphorylation levels at 60 kDa were maximal at gAd 20 μg/ml stimulation [Figure 3.14C].

PMA, a DAG analogue induced a strong increase in phosphorylation of PKC substrates with molecular weights of 140, 100, 80, 70, 60, 50, 30 and 25 kDa [Figure 3.14 and 3.15]. Importantly, the phosphorylation events induced by both gAd and PMA were blocked by the PKC inhibitor Ro 31-8220 [Figure 3.15]. The incubation of washed platelets with Ro 31-8220 (1-10 μM) reduced PKC substrate phosphorylation in a concentration-dependent manner [Figure 3.15B and C]. Ro 31-8220 (10 μM) was found to be the most effective concentration to inhibit the phosphorylation of PKC substrates. Taken together, this data in combination with previous aggregation data suggests that gAd activates a PKC isoform that plays a critical role in $\alpha_{IIb}\beta_3$ dependent aggregation, particularly at low gAd concentrations.

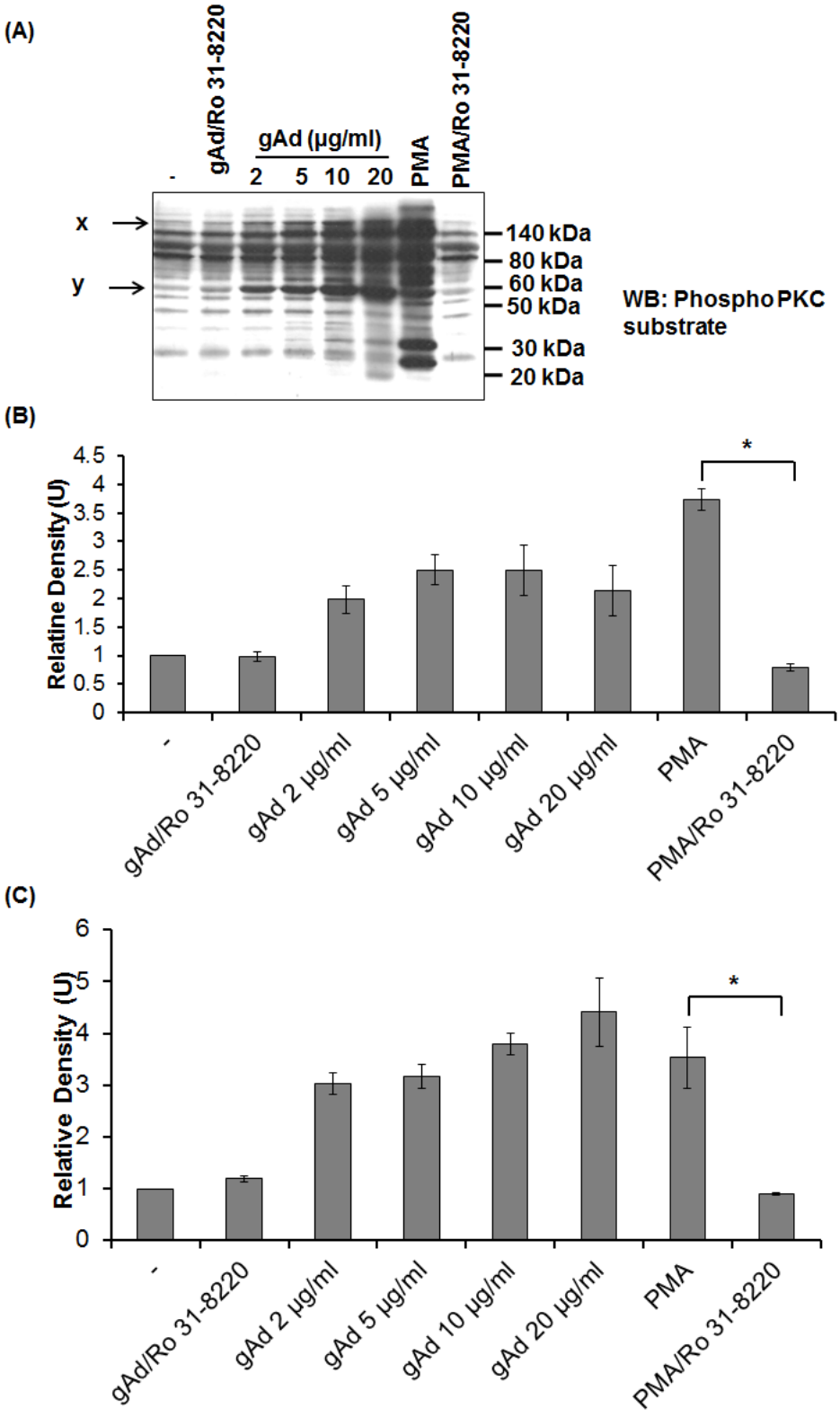


Figure 3.14: Globular adiponectin stimulates PKC substrate phosphorylation in a concentration-dependent manner. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with gAd (2-20 µg/ml) or PMA (300 nM) for 90 seconds, in the presence or absence of Ro 31-8220

(10 μM). Platelets were lysed, separated by SDS-PAGE and blotted with anti-phospho-PKC substrate antibody. (**B**) Densitometric measurement of phospho-PKC substrate at band labelled “x”. (**C**) As in (B) except band labelled “y” was selected and expressed as relative density to basal ± SEM of three independent experiments, where * indicates $p<0.05$ compared with PMA in the presence and absence of Ro 31-8220.

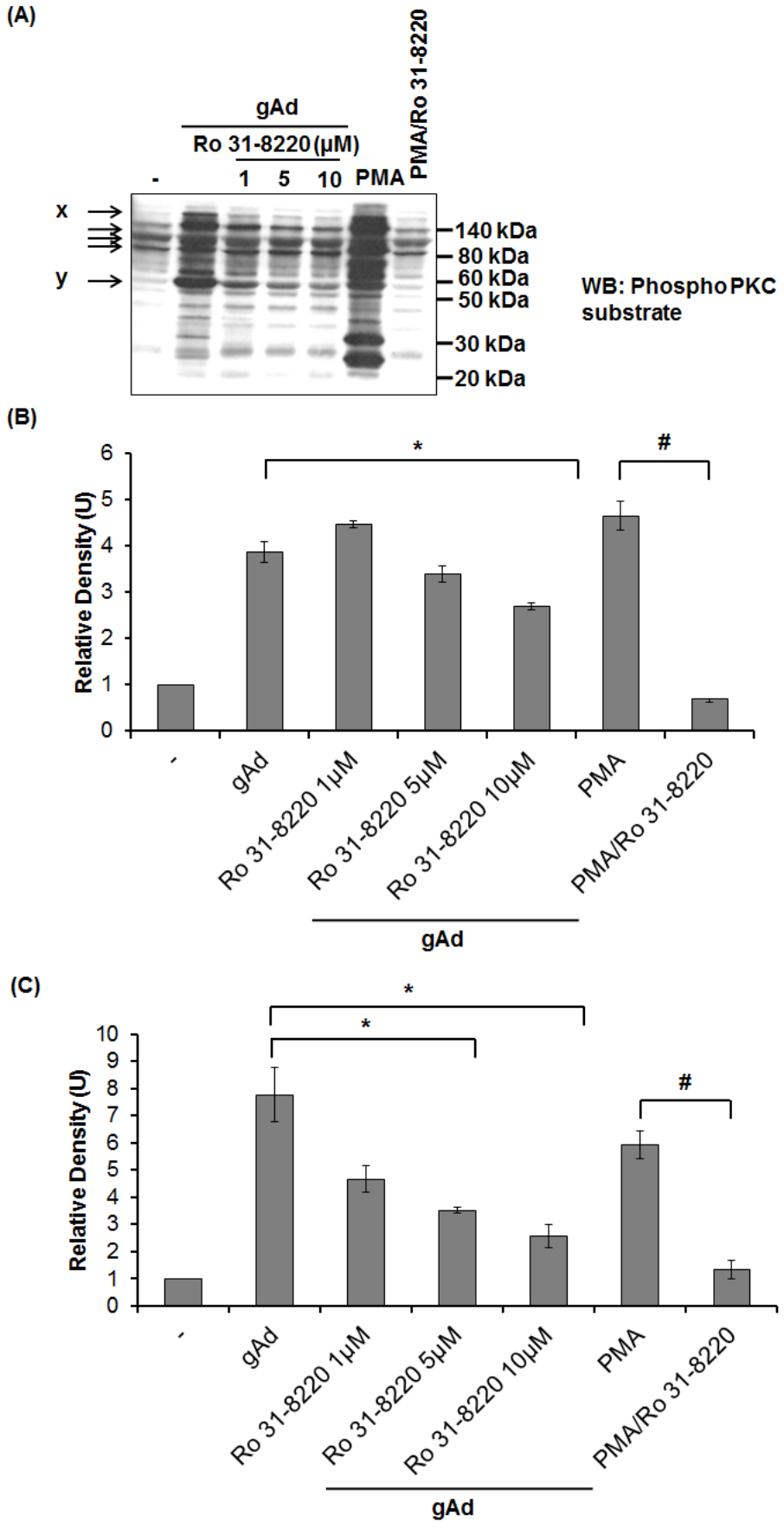


Figure 3.15: Globular adiponectin stimulates PKC activation. (**A**) Washed platelets (5x10^8 platelets/ml) were stimulated with gAd (20 μg/ml) or PMA

(300 nM) for 90 seconds, in the presence or absence of increasing concentration of Ro 31-8220 (1-10 μM). Platelets were lysed, separated by SDS-PAGE and blotted with anti-phospho-PKC substrate antibody. The arrows indicated gAd-stimulated PKC substrate protein phosphorylation. (**B**) Densitometric measurement of phospho-PKC substrate at band labelled "x". (**C**) As in (B) except band labelled "y" was selected and expressed as relative density to basal ± SEM of three independent experiments, where * indicates $p<0.05$ compared with PMA in the presence and absence of Ro 31-8220.

3.3.2 G-protein coupled receptors can compensate for the loss of PKC

Having established that gAd stimulated platelet aggregation occurred through PKC dependent and independent mechanisms, and that G-protein coupled receptors could synergise with gAd to maximise platelet aggregation, we investigated the mechanism that link these two observations. In the first instance we used aggregation to examine if G_i/G_z-coupled receptors could synergise with gAd to overcome the loss of PKC signalling. Platelet aggregation induced by gAd (20 µg/ml) was reduced from 60±5.1% to 28±3.8% in the presence of Ro 31-8220 ($p<0.05$) [Figure 3.16A and B]. However, the addition of ADP (5 µM) partially restored aggregation to 42±3.6% ($p<0.05$). This event occurred through the $P2Y_{12}$ coupled G_i receptor, since it was maintained in the presence of A3P5P. GAd-induced aggregation was also partially reversed by the addition of adrenaline acting through G_z-coupled α_{2A} adrenoceptor to 40±4.1% ($p<0.05$). It was possible that the synergism between adrenaline and gAd could lead to secretion of ADP, so these latter experiments were performed in the presence of apyrase [Figure 3.16A and B]. Since potentiation of aggregation still occurred in the presence of ADP antagonists the data indicated that the increased aggregation was due to a direct effect of adrenaline signalling and not an indirect effect of secreted ADP. Finally ADP was added in the presence of A3P5P and adrenaline to gAd stimulated platelets and under these conditions the aggregation response was fully reversed. These data indicate that G_i/G_z signalling may combine with gAd to stimulate aggregation independently of PKC.

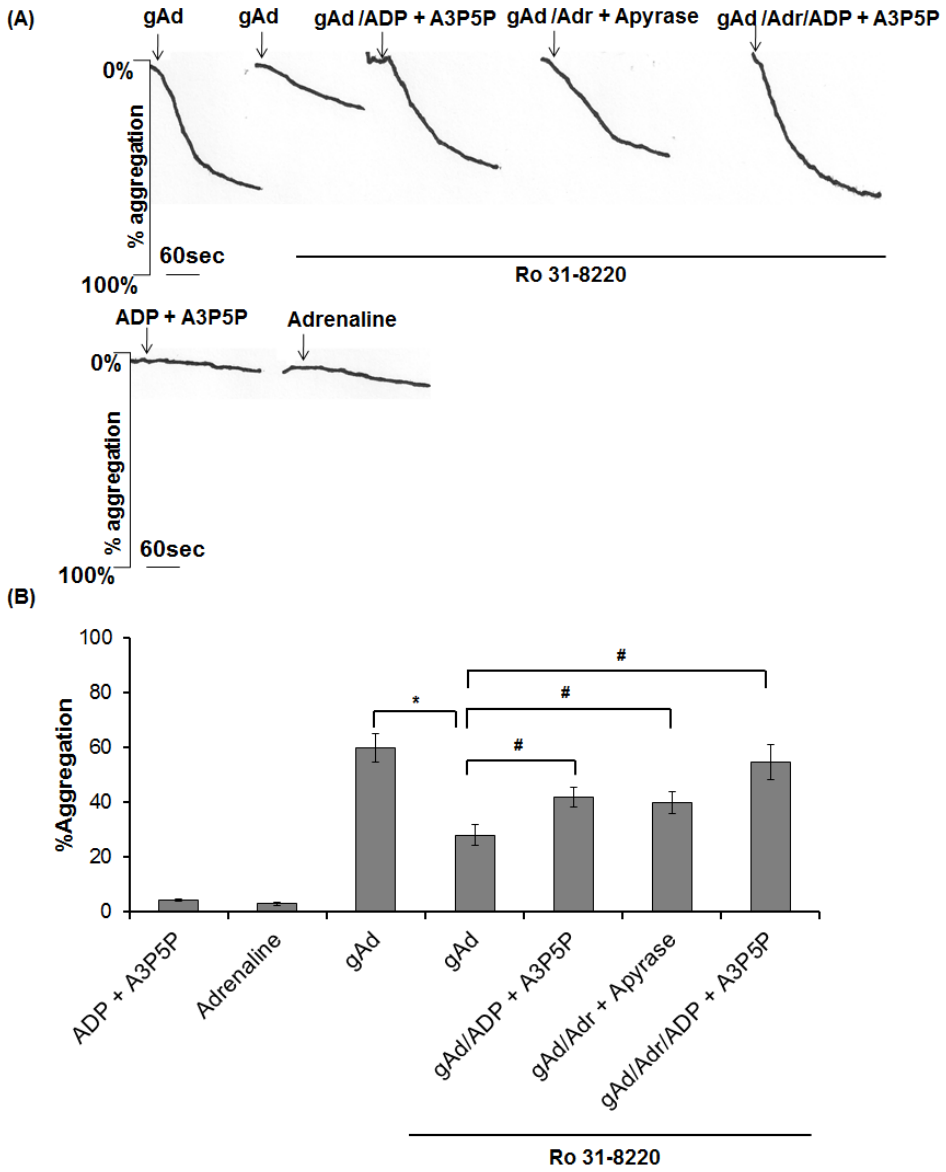


Figure 3.16: Recovery of platelet aggregation and secretion under conditions of PKC inhibition by G_i-coupled agonists. Washed platelets ($2.5x10^8$ platelets/ml) were pre-incubated with Ro 31-8220 (10 µM) and then stimulated with gAd (20 µg/ml) alone or premixed with ADP (5 µM), A3P5P (300 µM), adrenaline (10 µM) and apyrase (2 U/ml) and the aggregation response was monitored for 4 minutes under constant stirring. Stimulation of platelets with gAd in the absence of Ro 31-8220 was used as a control for these inhibitors. (**A**) Representative traces. (**B**) Collated data of three independent experiments expressed as mean ± SEM of aggregation, where * indicates $p<0.05$ compared with gAd in the presence and absence of Ro 31-8220 and # indicates $p<0.05$ compared with gAd/Ro 31-8220 in the presence and absence of ADP or adrenaline.

3.3.3 The role of dense-granule secretion in aggregation induced by globular adiponectin

While previous experiments indicate synergy between the primary pathways stimulated gAd and those by G_i/G_z, it was still unclear whether this required secretion. Using lumi-aggregation we found that gAd induced dense-granule secretion as exemplified by the release of 0.14±0.01 nM ATP, which was taken as 100% ATP secretion was ablated by Ro 31-8220 ($p<0.05$) [Figure 3.17], therefore demonstrating a key role for PKC in this process. To examine if ADP and adrenaline, which reversed the inhibitory effect on aggregation, restored secretion we added these agents to gAd stimulated platelets in the presence of Ro 31-8220. The addition of ADP (5 μM) under conditions of PKC inhibition led to a partially restoration in ATP secretion to 44.7±6.7% of the control value ($p<0.05$). This was not influenced by the presence of the $P2Y_1$ antagonist A3P5P [Figure 3.17], but was abolished by the $P2Y_{12}$ inhibitor MRS2395.

The secretion response was also partially restored by the addition of adrenaline to 72.9±3.3% ($p<0.05$) and fully restored by the combined addition of ADP and adrenaline ($p<0.05$). Importantly, this concentration of ADP (5 μM), in the presence of $P2Y_1$ receptor antagonist, and adrenaline (10 μM) failed to stimulate ATP secretion when tested independently of gAd [Figure 3.17]. Furthermore, the addition of wortmannin, a pan PI3K inhibitor was able to block gAd-stimulated secretion under PKC blockade and the addition of ADP and adrenaline ($p<0.05$) [Figure 3.17]. Taken together, these data suggest that compensatory pathways may stimulate granule secretion independent of PKC. The mechanism responsible for these effects is unclear, but may involve PI3K.

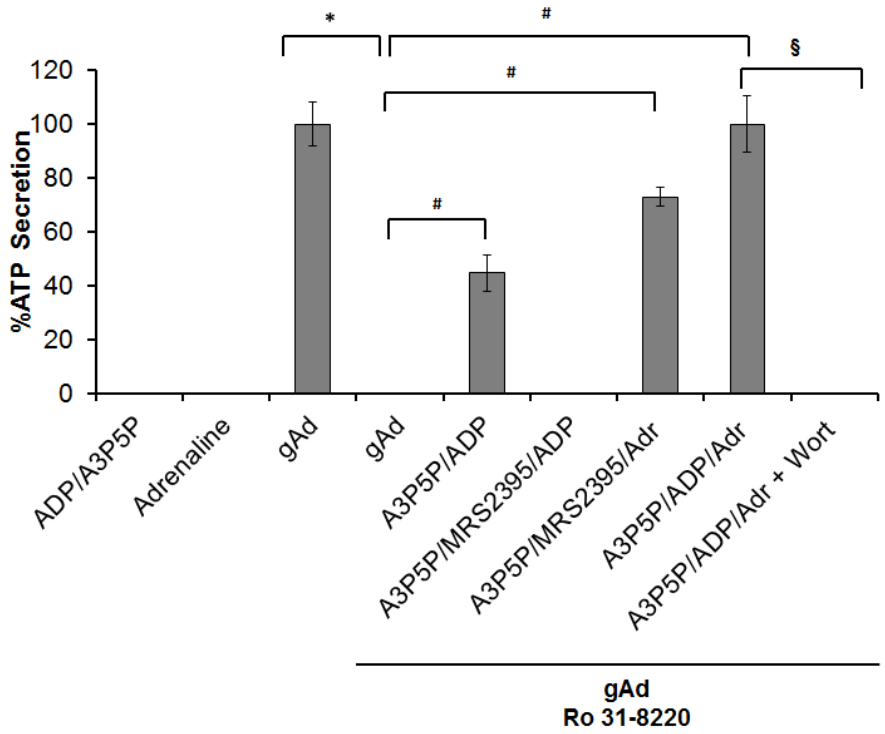


Figure 3.17: Restoration of dense-granule secretion by globular adiponectin by G_i-coupled agonists in platelets treated with PKC inhibitor. Washed platelets ($3x10^8$ platelets/ml) were pre-incubated with Ro 31-8220 (10 µM) and then stimulated with gAd (20 µg/ml) alone or in combination with ADP (5 µM), or adrenaline (10 µM). In some cases platelets were pre-treated with A3P5P (300 µM), apyrase (2 U/ml) or wortmannin (100 nM). The luminescence response was monitored for 4 minutes under constant stirring. Results were expressed as %ATP secretion ± SEM of three experiments, where * indicates $p<0.05$ compared with gAd stimulation in the presence of Ro 31-8220, # indicates $p<0.05$ for gAd/Ro 31-8220 compared to gAd/Ro 31-8220 in the presence of ADP or adrenaline, and § indicates $p<0.05$ for the presence and absence of wortmannin.

3.4 Discussion

Adiponectin is a major adipokine produced by adipose tissue involved in lipid and carbohydrate metabolism, although anti-diabetic and anti-inflammatory properties have been described (Okamoto et al., 2002, Ouchi and Walsh, 2007, Zhu et al., 2008). In addition to these, adiponectin has important vascular properties that can indirectly influence platelet function such as increasing the bioavailability of NO which is a potent platelet inhibitor (Naseem, 2005). However, the direct effect of adiponectin on platelet function is not very well understood. In this chapter the aim was to understand the role of both gAd and fAd on platelet aggregation and explore the mechanisms underpinning any functional effects observed.

Physiological significant concentrations of recombinant fAd did not stimulate platelet aggregation. In contrast, recombinant gAd was able to stimulate full irreversible platelet aggregation which was accompanied with platelet dense-granule secretion. Since recombinant forms of adiponectin expressed from *E.Coli* were used in these experiments, it was important to eliminate the possibility that potential endotoxin contamination could stimulate platelet aggregation. Riba et al., (2008) showed that similar levels of endotoxin found in the recombinant adiponectin (2 ng) do not induce platelet aggregation (Riba et al., 2008). It was also important to consider that post-translation modifications differ from bacterial to eukaryote systems which can influence its biological activity (Wang et al., 2008). The same work by Riba et al., (2008) showed that fAd expressed from HEK cells do not stimulate aggregation and importantly, gAd stimulated similar levels of platelet aggregation to that of the recombinant form of the protein. Taken together, this data suggests that only the globular form of adiponectin is able to stimulate platelet aggregation. This is surprising since fAd contains the GPVI-specific GPO motifs within the collagen-like domain. It could be possible that the platelet collagen receptors cannot access the collagen-like domain of the fAd monomer due to steric hindrance. In addition to this, fAd also contains the globular domain but was unable to stimulate aggregation.

Having identified that only globular form of adiponectin can induce aggregation, it was important to understand the signalling pathways involved. The same study by Riba et al., (2008) suggested that gAd activated platelet through a tyrosine kinase-linked mechanism that was linked to GPVI. However, the role of other platelet activators and signalling enzyme was not studied and required clarification.

GAd was found to stimulate integrin $\alpha_{IIb}\beta_3$ dependent aggregation through pathways dependent and independent of PKC activation. It is now widely recognised that PKC isoforms are involved in the release of platelet derived secondary mediators such as ADP and TxA_2 (Harper and Poole, 2010). Taking this information in conjunction with previous data indicating that gAd-mediated aggregation was tyrosine kinase-dependent, experiments were designed to create a more detailed model for aggregation induced by gAd.

Consistent with the study by Riba et al., (2008) using an inhibitor of Src family kinases showed that platelet aggregation was tyrosine kinase-dependent and gAd induced the phosphorylation of numerous proteins. Some of these have been identified as Syk, PLCγ2 and LAT (Riba et al., 2008). Since these data suggest a collagen type mechanism, this was used as a model with which to understand gAd-stimulated aggregation. Collagen-induced aggregation proceeds via two phases. The first phase is the activation of the platelets via a tyrosine kinase dependent mechanism which enables integrin $\alpha_{IIb}\beta_3$ dependent aggregation by inside-out signalling (Calvete, 1995, Shattil, 1999). The second phase involves the release of platelet-derived soluble mediators such as ADP and TxA_2 through pathways linked to PKC activation. In the first instance the effect of platelet-derived soluble mediators on gAd-stimulated tyrosine phosphorylation was examined. Under non-aggregatory conditions, the tyrosine phosphorylation of platelet proteins in whole cell lysates were not affected by either inhibition of endogenous ADP and TxA_2 signalling or the addition of exogenous ADP or U46619. This is consistent with a mechanism whereby gAd induces direct receptor mediated tyrosine phosphorylation.

In contrast, maximal aggregation induced by higher concentrations of gAd showed a critical requirement for the co-secretion of both ADP and TxA_2. Interestingly, the loss of ADP signalling was found to be less important than the loss of TxA_2. Aggregation was reduced modestly by the antagonism of ADP receptors, but was reduced by approximately 50% due to the loss of TxA_2 signalling. Interestingly, the combination of the inhibitors caused a further reduction in aggregation suggesting that both secondary agonists played a role in inducing maximal aggregation. In contrast, at lower concentrations the adipokine had an absolute requirement for secondary signalling. This led to the possibility that secondary mediators can potentiate gAd-stimulated aggregation, particularly at lower concentrations. Critically, ADP was able to synergise with threshold concentrations of gAd to potentiate aggregation. This may have pathophysiological implications since it would suggest that low levels of this plasma protein may be able to enhance the effects of physiological activators of platelets.

The presence of A3P5P, a $P2Y_1$ ADP receptor antagonist, had no effect on the ability of ADP to enhance gAd-mediated platelet aggregation. Conversely, MRS2395, a selective antagonist of the $P2Y_{12}$ ADP receptor coupled to G_i abolished the ability of ADP to influence aggregation suggesting strongly that G_i played a critical role in the potentiation of aggregation. Furthermore, stimulation of the α_{2A}-adrenoceptor coupled to G_z, could overcome the inhibitory effects of ADP receptor antagonists. In contrast, thromboxane TPα/β receptor coupled to G_q exhibited weak synergy with gAd, despite indomethacin exerting a strong inhibitory effect on aggregation. These observations indicate that a G_i/G_z-dependent pathway initiated by either ADP or adrenaline participates in gAd-stimulated platelet aggregation. However, it was difficult to reconcile why indomethacin could block aggregation but U46619 did not potentiate submaximal aggregation induced by gAd. One possibility was that TxA_2 signalling was restored by ADP secretion and that the two worked concurrently rather than simultaneously. This view was supported by

evidence from (Li et al., 2003) who demonstrated that TxA_2 may induce secretion of ADP in two waves. When dense-granule secretion was examined it was found that gAd-induced dense-granule secretion was abolished by indomethacin, but remained unaffected by blocking the ADP receptors. Critically, the ablation of dense-granule secretion by indomethacin is partially reversed by the TxA_2 analogue U46619. These data suggest that gAd stimulated secretion of ADP was driven at least in part by TxA_2, which when released was able to synergize with primary signalling from gAd.

Early aggregation experiments determined that secretion of platelet granules was required for maximal aggregation. Since PKC isoforms play a major role in platelet granule secretion, we examined the role of PKC in this process (Gerrard et al., 1989, Murugappan et al., 2004, Harper and Poole, 2007). GAd stimulated the phosphorylation of PKC substrates suggest that the kinase was activated downstream of the adipokine. The use of the cell-permeable inhibitor of PKC, Ro 31-8220, provided an insight into the pathways used by gAd to stimulate aggregation. Under conditions that block the activity of PKC, we found that platelet aggregation in response to gAd was reduced but not abolished, indicating that aggregation could proceed through PKC-dependent and independent pathways. Indeed that loss of PKC produced an aggregation response similar to that found in the absence of ADP or TxA_2. GAd-mediated and U46619 mediated dense-granule secretion were ablated by PKC inhibitors. Taken together this suggested a model whereby gAd may stimulate PKC mediated generation of TxA_2, which in turn drove dense-granule secretion and maximal platelet aggregation.

Interestingly, diminished platelet aggregation associated with PKC inhibition could be overcome by the addition of ADP or adrenaline. Here we found that ADP signalling required $P2Y_{12}$ but not $P2Y_1$, while adrenaline could potentiate aggregation directly without the need of ADP. Thus G_i/G_z signalling can potentiate platelet aggregation independently of PKC. This data suggest that these compensatory pathways may occur through their

ability to stimulate secretion independently of PKC. The mechanism responsible for these effects is unclear, but may involve either reduced activity of PI3K or adenylyl cyclase, since G_i is coupled to both adenylyl cyclase and a downstream activation of PI3K (Murugappa and Kunapuli, 2006). To date G_i has a very limited ability to reduce basal cAMP levels (Yang et al., 2002), and is most effective in the presence of agents that stimulate the activity of adenylyl cyclase. Thus, it is unlikely that the gAd-mediated synergism with G_i-coupled receptors occur at the level of adenylyl cyclase. We therefore examined how secondary signalling induced by gAd influenced PI3K. Platelet dense-granule secretion induced by the addition of exogenous ADP, in the absence of PKC activity, was blocked by the pan PI3K inhibitor wortmannin. However, it was unclear which isoform is responsible since both PI3Kβ and PI3Kγ are activated downstream of ADP G_i-coupled $P2Y_{12}$ receptor and contribute to integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation (Hirsch et al., 2001, Jackson et al., 2005, Lian et al., 2005). While more recently, Kim and colleagues (Kim et al., 2009) have demonstrated that GPVI mediated secretion, at least in part, requires G_i-stimulated of PI3Kβ. Thus it is possible that G_i/G_z signalling is required for pathways that drive platelet secretion independently of PKC. Taken together, these data indicate that gAd-stimulated secondary signalling can potentiate platelet activation via the up-regulation of TxA_2 formation which can further drive ADP release from platelet dense-granules and in combination with more established agents can contribute to platelet aggregation at sites of vascular injury [Figure 3.18].

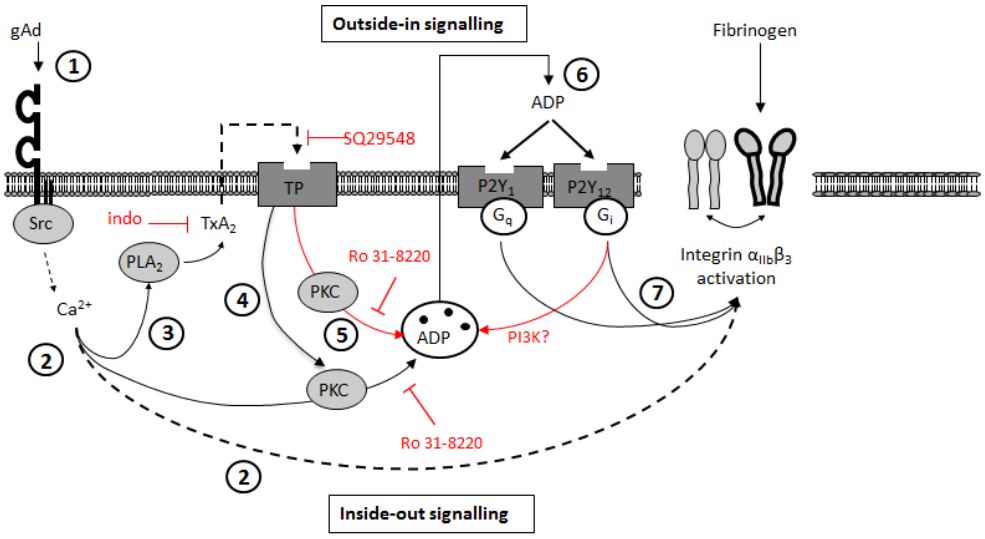


Figure 3.18: The proposed role of secondary mediators in globular adiponectin-stimulated aggregation. Platelets are stimulated with gAd through a tyrosine kinase-dependent mechanism **(Step 1)** leading to increased calcium mobilisation and activation of integrin $\alpha_{IIb}\beta_3$ by inside-out signalling **(Step 2)**. TxA_2 formation is up-regulated by PLA_2 activation **(Step 3)**, which drives ADP release from platelet dense-granules **(Step 4)** and further potentiating platelet aggregation by outside-in signalling **(Step 5)**. However, signals from G_i/G_z activation **(Step 6)** can further potentiate platelet aggregation **(Step 7)** through the release of platelet dense-granules via PKC independent pathways that may include PI3K.

Chapter 4: The role of PI3K in globular adiponectin-stimulated aggregation

4.1 Introduction

In chapter 3 it was established that only the globular form of adiponectin stimulates platelet aggregation in a tyrosine kinase-dependent mechanism. Furthermore, the release of platelet derived mediators such as ADP and TxA_2 was required to achieve maximal aggregation. In addition to this we found gAd can synergise with G_i/G_z mediated signalling to induce aggregation and secretion independent of PKC signalling. The addition of the PI3K inhibitor, wortmannin seemed to abolish the ability of gAd to induce PKC-independent secretion. These data strongly suggest a prominent role for PI3K in this process. In the present chapter the aim was to further understand the role of PI3K in gAd-stimulated aggregation and secretion.

The phosphorylation of the D3-hydroxy-group of the inositol ring of phosphoinositides was first described in 1988 (Whitman et al., 1988). The exploration of these new phosphoinositides products have led to the discovery of a new group of enzymes called PI3K (Carpenter et al., 1990, Cantley, 2002). Studies have shown that PI3K plays an important role in many different cell types in regulating their function. These include haemopoietic cells such as neutrophils, monocytes and B- and T-cells (Koyasu, 2003). Three classes of PI3K have been described, which are grouped according to their structure and preference of substrate (Fruman et al., 1998, Vanhaesebroeck and Waterfield, 1999). Platelets are found to express two classes of PI3K including class I, which is further divided to class IA (PI3Kα, PI3Kβ and PI3Kδ) and class IB (PI3Kγ), and class II (PI3KC2α) (Vanhaesebroeck and Waterfield, 1999). Class IA PI3K isoforms consist of p110 catalytic subunit and is associated with p85/p55 regulatory subunit that contains SH2 domains (Fruman et al., 1998). These SH2 domains allow the binding of tyrosine phosphorylated proteins that can help to activate the enzyme (Bibbins et al., 1993). The p85/p55 regulatory subunits also

contain a proline rich region that allows the binding of SH3 containing proteins such as Fyn and Lyn which can increase PI3K activation by 5 to 7 fold (Pasquet et al., 1999). Class IB PI3K isoform is also composed of the p110 catalytic subunit, but it is associated with a p101 regulatory subunit (Fruman et al., 1998). The class IB p110 catalytic subunit can be distinguished from class IA by its ability to bind to βγ subunits of heterotrimeric G-proteins. Furthermore, class IB PI3K isoform is not activated by tyrosine phosphorylated proteins but by βγ subunits in G-protein coupled receptor signalling (Stephens et al., 1997).

The activation of PI3K isoforms have been implicated in many platelet responses that include platelet shape change and aggregation (Rittenhouse, 1996, Pasquet et al., 1999). The type of PI3K isoform activated is dependent on the type of receptor activated, indicating that different isoforms are linked to distinct receptor mediated pathways. Platelet adhesion to exposed sub-endothelial proteins such as vWF is a critical step in the formation of a haemostatic plug. The activation of PI3Kα and PI3Kβ isoforms downstream of GPIb-V-IX receptor complex activation has been reported to play an important role in this process. The inhibition of these PI3K isoforms by pharmacological inhibitors can inhibit vWF induced integrin $\alpha_{IIb}\beta_3$ activation (Yap et al., 2002). Platelet activation by soluble platelet agonists such as ADP and thrombin which signal through G-protein coupled receptors are often coupled to class I PI3K isoforms (Jackson et al., 2005, Kim et al., 2009, Kim and Kunapuli, 2011). These PI3K isoforms are involved in signalling pathways that enable the activation of the integrin $\alpha_{IIb}\beta_3$ by inside-out signalling. The activation of G_i-coupled ADP $P2Y_{12}$ receptor is of particular importance since it can potentiate platelet aggregation through pathways linked to PI3Kβ and PI3Kγ activation (Jackson et al., 2005, Kim et al., 2009, Garcia et al., 2010, Kim and Kunapuli, 2011). There is compelling evidence that PI3K is involved in outside-in signalling downstream of integrin $\alpha_{IIb}\beta_3$ activation (Zhang et al., 1998). Platelet adhesion to fibrinogen has provided direct evidence of the importance of PI3K isoforms in this process. The accumulation of

phosphatidylinositol 4,5-bisphosphate (PIP_2) downstream of integrin $\alpha_{IIb}\beta_3$ activation via adhesion to fibrinogen is a critical event in Ca^{2+} mobilisation and lamellipodia formation (Gironcel et al., 1996). The PI3K isoforms involved in integrin $\alpha_{IIb}\beta_3$ activation is less clear but studies have shown that the class II PI3KC2α isoform is involved (Banfić et al., 1998, Zhang et al., 1998).

Therefore, the present study set out to define more precisely the role of PI3K in gAd-mediated platelet activation and which isoforms were responsible.

4.2 The role of PI3K in globular adiponectin-stimulated aggregation

We have established that gAd is a novel platelet agonist and shown that aggregation proceeds through pathways that require activation of PKC and PI3K [Figure 3.2]. The role of PI3K was further elaborated in gAd-stimulated aggregation using a pan PI3K inhibitor, wortmannin. This inhibitor can compete with ATP for binding to the PI3K catalytic domain and blocks the catalytic site via a covalent interaction with a critical lysine 802 residue and therefore inhibiting the kinase (Wymann et al., 1996).

Aggregation induced by gAd (20 μg/ml) was almost completely abolished by treating the platelets with wortmannin (100 nM). Under these conditions aggregation was reduced from 61±7.6% to 5±2.8% ($p<0.05$) [Figure 4.1A]. This inhibition of aggregation was accompanied by the complete loss of ATP secretion ($p<0.05$) [Figure 4.2]. To understand the contribution of different PI3K isoforms in platelet aggregation in response to different platelet agonists, these experiments were repeated with collagen, CRP-XL and thrombin. Platelet aggregation and secretion induced by collagen (10 μg/ml) was found to be completely abolished in the presence of wortmannin ($p<0.05$) [Figure 4.1C and 4.2]. Similarly, we found aggregation induced by CRP-XL (10 μg/ml) in the presence of wortmannin was almost completely abolished and this was associated with complete inhibition of secretion [Figure 4.1B and 4.2]. In contrast, stimulation of platelets with a non-GPVI agonist, thrombin (0.1 U/ml) in the presence of wortmannin only had a modest effect on aggregation, reducing it from 66±7.2% to 43±7.3%, however, we found secretion to be completely abolished under these conditions ($p<0.05$) [Figure 4.1D and 4.2]. Interestingly, increasing thrombin concentration to 0.5 U/ml in the presence of wortmannin failed to ablate aggregation (86±3.6% to 78±4.3%) and only had a modest effect on thrombin-stimulated ATP secretion (1.46±0.1 nM to 1.14±0.1 nM) [Figure 4.1E and 4.2], consistent with PI3K dependent and independent mechanism of thrombin-induced aggregation (Holinstat et al., 2009). Taken together, these data suggest that PI3K plays a critical role in both platelet aggregation and secretion, particularly in

response to agonist that stimulates activation through a tyrosine kinase-dependent mechanism.

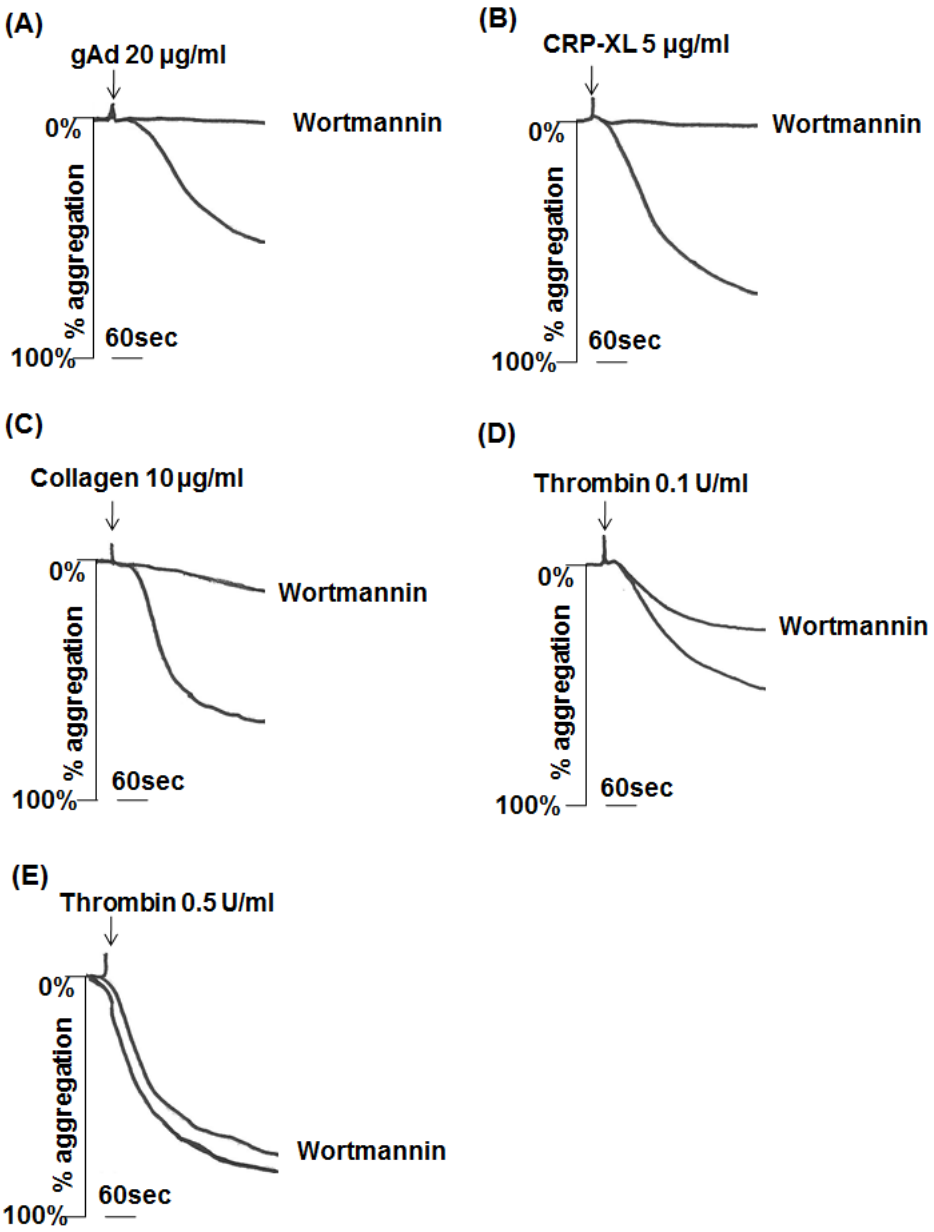


Figure 4.1: The effect of PI3K inhibition on globular adiponectin-stimulated aggregation. Washed platelets ($3x10^8$ platelets/ml) were stimulated with (**A**) gAd (20 µg/ml) (**B**) CRP-XL (5 µg/ml) (**C**) collagen (10 µg/ml) (**D**) thrombin (0.1 U/ml) (**E**) thrombin (0.5 U/ml) in the absence and presence of wortmannin (100 nM) and the aggregation response was monitored for 4 minutes under constant stirring. All representative aggregation traces are taken from four independent experiments performed with distinct blood donors.

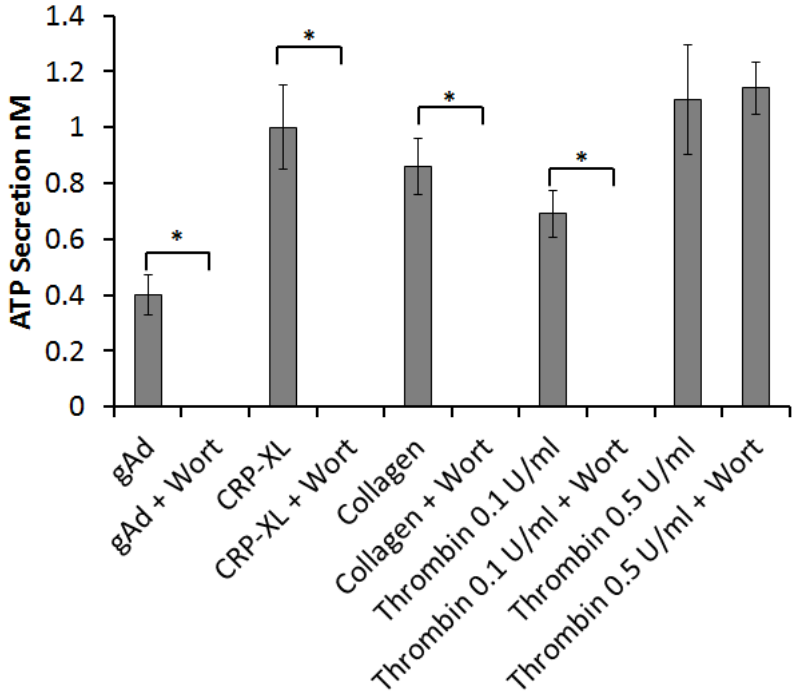


Figure 4.2: The effect of PI3K inhibition on globular adiponectin-stimulated secretion. Washed platelets ($3x10^8$ platelets/ml) were stimulated with gAd (20 µg/ml), CRP-XL (5 µg/ml), collagen (10 µg/ml) and thrombin (0.1-0.5 U/ml) in the absence and presence of wortmannin (100 nM) and the luminescence response was monitored for 4 minutes under constant stirring and results were expressed as ATP nM secretion ± SEM of four independent experiments, where * indicates $p<0.05$ compared with the agonist alone to wortmannin.

4.2.1 Identification of PI3K isoforms in globular adiponectin-stimulated aggregation and secretion

It is established that multiple isoforms of PI3K are expressed in platelets and we wished to examine the role of the individual isoforms in platelet activation induced by gAd (Vanhaesebroeck and Waterfield, 1999). This was achieved by using selective PI3K isoform inhibitors including PI3Kβ inhibitor TGX-221, PI3Kα inhibitor PIK75 and PI3Kγ inhibitor AS242525 (Sadhu et al., 2003, Knight et al., 2006, Kim et al., 2007, Gilio et al., 2009, Kim et al., 2009). Here, these same inhibitors were used to identify the PI3K isoform activated in response to gAd.

Treatment of gAd-stimulated (20 μg/ml) platelets with TGX-221 (500 nM) (Kim et al., 2009) caused a significant inhibition of aggregation, where aggregation was reduced from 65±6.2% to 10±0.7% ($p<0.05$) [Figure 4.3A]. Similarly, the PIK75 (100 nM) (Kim et al., 2009) partially inhibited aggregation to 19±5.5% ($p<0.05$) [Figure 4.3A]. In contrast, platelets treated with AS242525 (100 nM) (Kim et al., 2009) showed minor effect on gAd-stimulated aggregation that was not significant [Figure 4.3A]. These data suggest that the PI3Kα and PI3Kβ isoforms play a significant role in gAd-stimulated aggregation.

To determine if these PI3K isoforms contribute to gAd-stimulated secretion, ATP secretion was measured by lumi-aggregation in the presence of these selective PI3K isoform inhibitors. Consistent with the aggregation data [Figure 4.3A], TGX-221 (500 nM) and PIK75 (100 nM) abolished gAd-stimulated dense-granule secretion [Figure 4.3B]. The PI3Kγ inhibitor had a minor and non-significant effect on gAd-stimulated secretion. Here ATP release was reduced from 0.32±0.1 nM to 0.22±0.1 nM [Figure 4.3B]. These data suggest a mechanism whereby PI3K isoforms α and β are activated downstream of gAd activation which contribute to platelet aggregation and secretion.

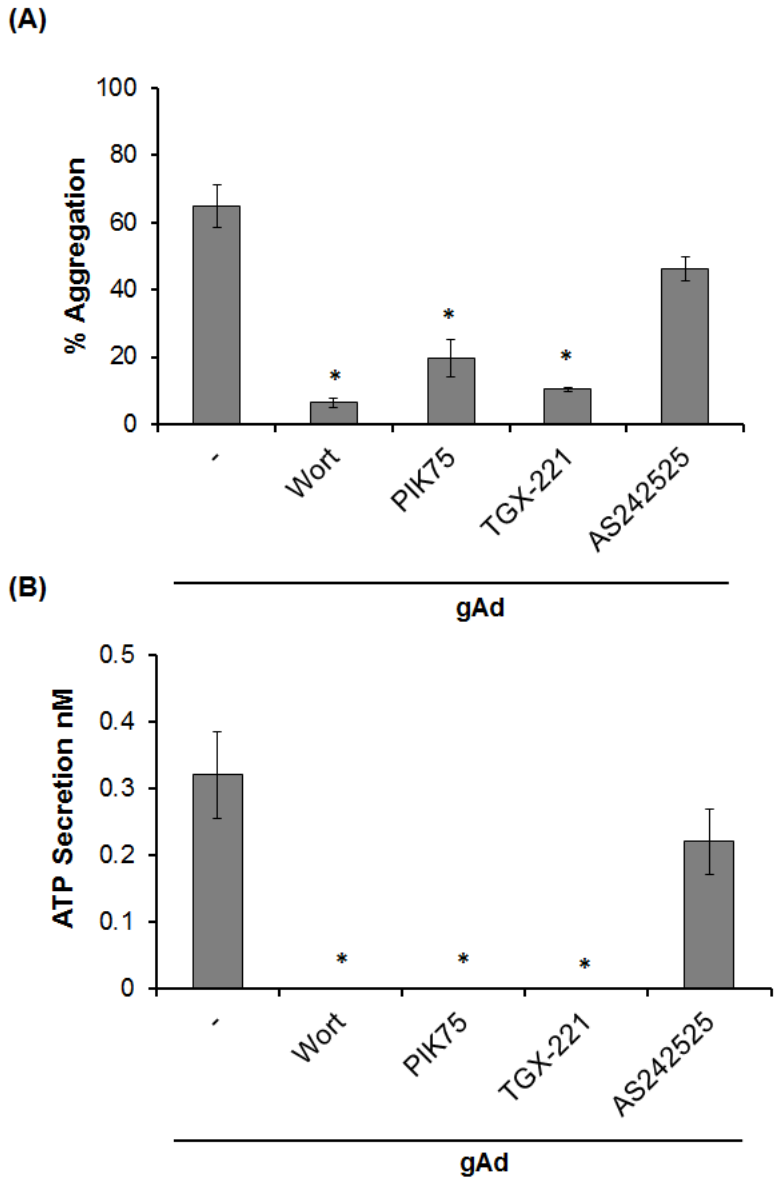


Figure 4.3: The role of PI3K isoforms on globular adiponectin-stimulated aggregation and secretion. (**A**) Washed platelets (3x10^8 platelets/ml) were stimulated with gAd (20 µg/ml) in the absence and presence of wortmannin (100 nM), PIK75 (100 nM), TGX-221 (500 nM), AS242525 (100 nM) and the aggregation response was monitored for 4 minutes under constant stirring and results were expressed as %Aggregation ± SEM of three independent experiments. (**B**) As in (A) but the luminescence response was monitored for 4 minutes under constant stirring and results were expressed as ATP nM secretion ± SEM of three independent experiments, where * indicates $p<0.05$ compared with gAd alone to the inhibitors.

To determine if these same PI3K isoforms are involved in other agonist induced platelet aggregation, which signal via a tyrosine kinase mechanism, the previous experiment were repeated with a GPVI selective agonist, CRP-XL and collagen. Here we found that TGX-221 exerted a strong inhibitory response on CRP-XL (10 μg/ml)-stimulated platelets, reducing aggregation from 76±3.1% to 13±3.7% ($p<0.05$) [Figure 4.4A]. In contrast, PIK75 exerted a modest effect in comparison, reducing aggregation to 53±5.4% ($p<0.05$) [Figure 4.4A]. This was accompanied with the complete loss of ATP secretion with TGX-221 stimulation ($p<0.05$). Similarly, PIK75 significantly reduced ATP secretion from 1.05±0.1 nM to 0.35±0.2 nM ($p<0.05$) [Figure 4.4B]. The PI3Kγ isoform inhibitor had only a minor effect on CRP-XL-stimulated aggregation or secretion [Figure 4.4B]. A similar dependency of PI3Kβ and α was observed in collagen-stimulated (10 μg/ml) platelets. Here aggregation was reduced from 63±6.1% to 23±9.6% by PIK75 ($p<0.05$) and almost completely abolished with TGX-221 ($p<0.05$) [Figure 4.5A]. Once again inhibition of PI3Kγ only had a minor effect on aggregation and secretion [Figure 4.5]. In contrast, inhibition of PI3Kβ and α completely abolished collagen-stimulated secretion at 10 μg/ml [Figure 4.5B]. Taken together, these data highlight the important roles of PI3K isoforms α and β in GPVI-stimulated aggregation and secretion.

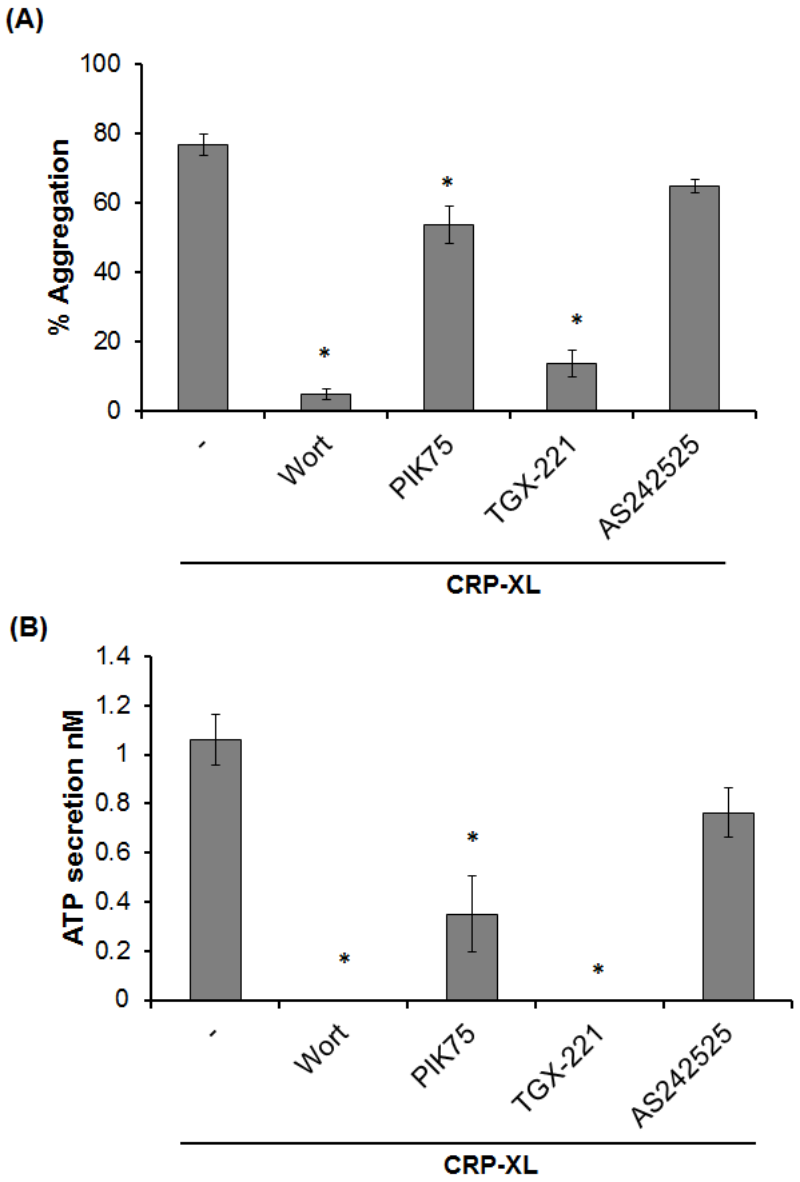


Figure 4.4: The role of PI3K isoforms in CRP-XL-stimulated aggregation and secretion. (**A**) Washed platelets ($3x10^8$ platelets/ml) were stimulated with CRP-XL (10 µg/ml) in the absence and presence of wortmannin (100 nM), PIK75 (100 nM), TGX-221 (500 nM), AS242525 (100 nM) and the aggregation response was monitored for 4 minutes under constant stirring and results were expressed as %Aggregation ± SEM of three independent experiments. (**B**) As in (A) but the luminescence response was monitored for 4 minutes under constant stirring and results were expressed as ATP nM secretion ± SEM of three independent experiments, where * indicates $p<0.05$ compared with CRP-XL alone to the inhibitors.

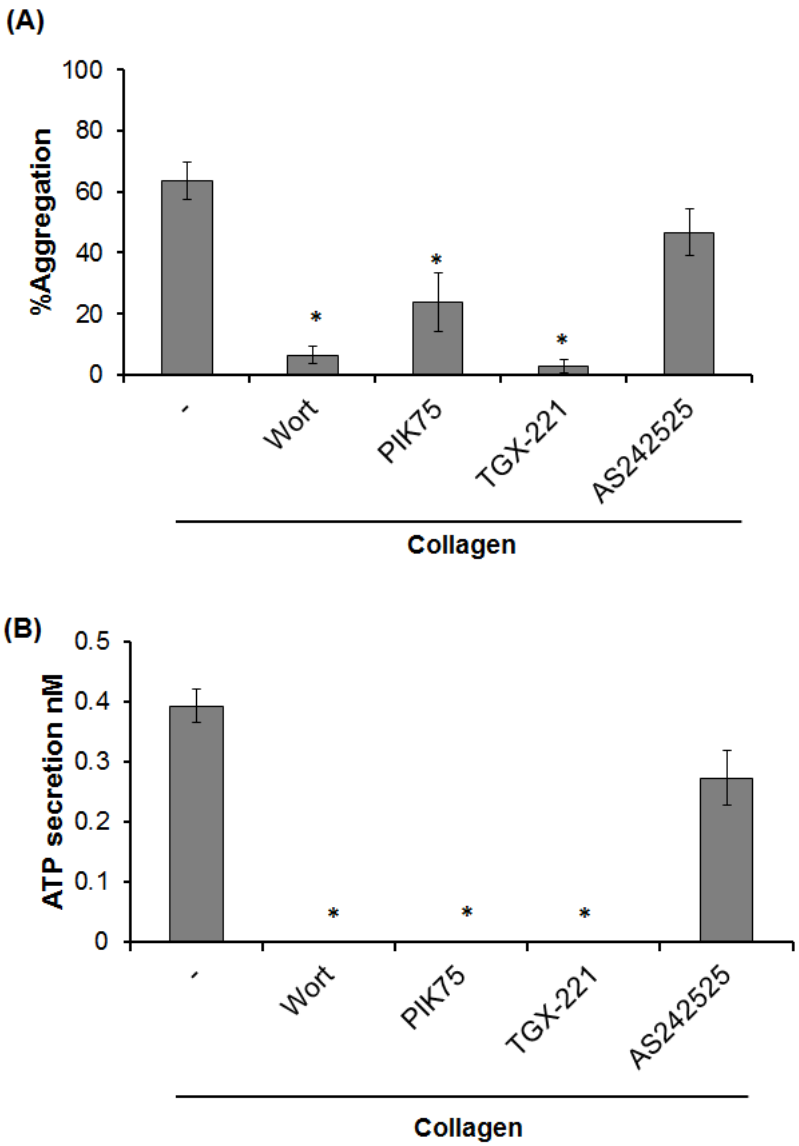


Figure 4.5: The role of PI3K isoforms in collagen-stimulated aggregation and secretion. (**A**) Washed platelets (3x10^8 platelets/ml) were stimulated with collagen (10 µg/ml) in the absence and presence of wortmannin (100 nM), PIK75 (100 nM), TGX-221 (500 nM), AS242525 (100 nM) and the aggregation response was monitored for 4 minutes under constant stirring and results were expressed as %Aggregation ± SEM of three independent experiments. (**B**) As in (A) but the luminescence response was monitored for 4 minutes under constant stirring and results were expressed as ATP nM secretion ± SEM of three independent experiments, where * indicates $p<0.05$ compared with collagen alone to the inhibitors.

4.3 The effect of PI3K inhibition on globular adiponectin-stimulated tyrosine phosphorylation

Since our previous data suggests that gAd stimulates platelets through a tyrosine kinase-dependent manner, we examined the role of PI3K on tyrosine phosphorylation. Consistent with our previous data [Figure 3.3], gAd (20 μg/ml) induced a robust increase in tyrosine phosphorylation of a number of proteins with prominent bands at 45, 50, 70, 125 and 145 kDa [Figure 4.6]. The presence of the PI3K inhibitor, wortmannin had no effect on tyrosine phosphorylation levels [Figure 4.6], suggesting that PI3K plays a critical role in both platelet aggregation and secretion through pathways independent of tyrosine phosphorylation.

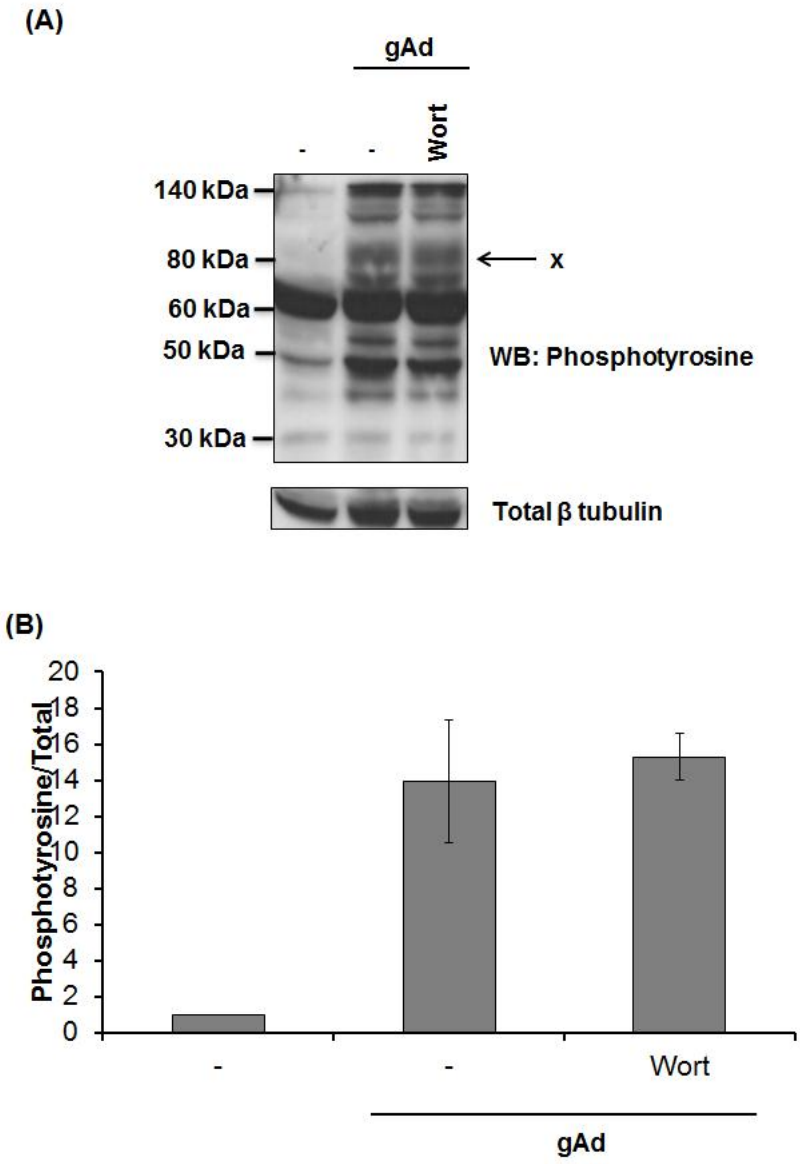


Figure 4.6: The effect of PI3K inhibition on globular adiponectin-stimulated tyrosine phosphorylation. (**A**) Washed platelets ($5x10^8$ platelets/ml) were incubated with apyrase (2 U/ml), indomethacin (10 µM) and EGTA (1 mM) for 20 minutes and then stimulated with gAd (20 µg/ml) in the presence and absence of wortmannin (100 nM) for 90 seconds. Proteins were resolved on 10-18% gradient polyacylamide gel SDS-PAGE and immunoblotted using anti-phosphotyrosine antibody. (**B**) Densitometric measurement of phosphotyrosine at band labelled "x" , expressed as phosphotyrosine/total ± SEM of three independent experiments.

4.4 The role of PI3K and Akt signalling pathway in globular adiponectin-stimulated platelets

4.4.1 Optimisation of Akt phosphorylation antibody

To understand the signalling events underlying PI3K activation in gAd-stimulated aggregation and secretion, Akt a downstream marker of PI3K activity was chosen. Akt is a 60 kDa serine–threonine kinase that is phosphorylated at two different residues, threonine^{308} and serine^{473} by phosphoinositide-dependent kinase (PDK) 1 and PDK2 respectively, and contributes to platelet activation (Wick et al., 2000, Cantley, 2002, Hresko and Mueckler, 2005). However, Akt phosphorylation also depends upon the upstream activation of PI3K and the synthesis of phosphatidylinositol, therefore the changes in Akt phosphorylation can be used to investigate PI3K mediated signalling.

An anti-Akt serine^{473} antibody was used to investigate the role of Akt in gAd-stimulated platelet activation. In the first instance, the optimum conditions for the antibody were determined. Washed platelets were stimulated with collagen (80 µg/ml) and an equal volume of 2xLaemelli buffer was added. The proteins were resolved on a 10% resolving gel, transferred to a PVDF membrane, blocked with 10% BSA and subjected to immunoblotting. As shown in figure 4.7A, the anti-Akt serine^{473} antibody bound to several proteins at 50, 80 kDa, including Akt at 60 kDa. To reduce the non-specific binding to these proteins, the blocking conditions were varied. The reduction of blocking buffer from 10% BSA to 5% BSA increased some of the non-specific binding at 70, 100 and 140 kDa [Figure 4.7C]. To further optimise the blocking conditions of the antibody, the membrane was blocked with milk protein. Interestingly, blocking with 10% milk protein reduced most of the non-specific binding compared to BSA, however, a small band was observed at 50 kDa [Figure 4.7B]. Further reduction of milk protein to 5% completely abolished all non-specific binding and a prominent Akt serine^{473} phosphorylation band was observed at 60 kDa [Figure 4.7D].

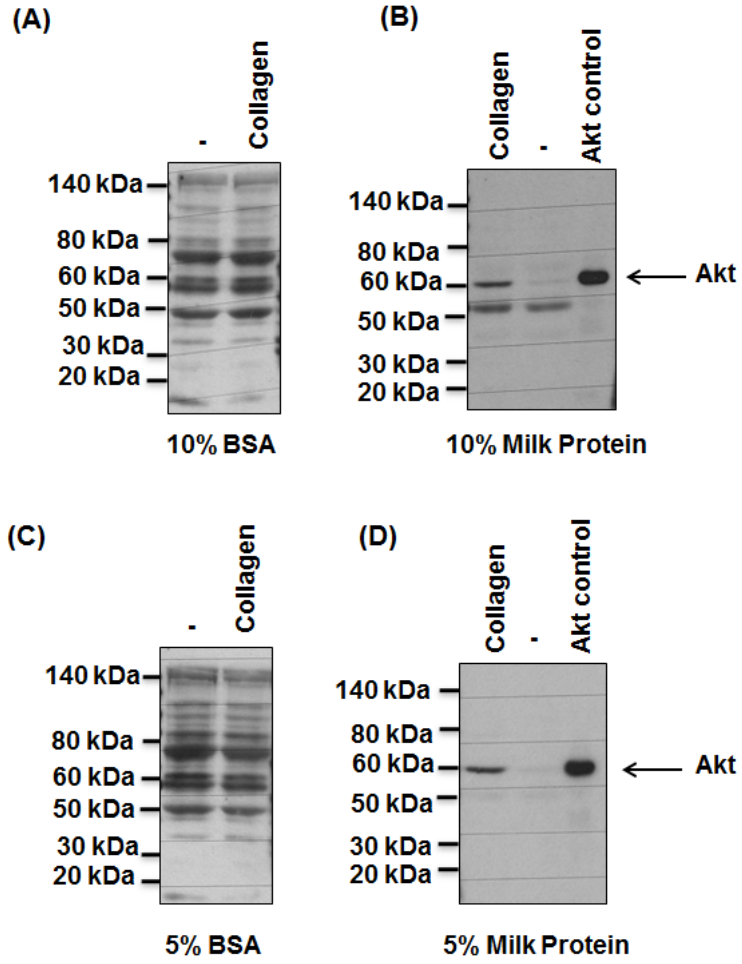


Figure 4.7: Optimisation of Akt serine^{473} phosphorylation antibody conditions. Washed platelets (5x10^{8} platelets/ml) were stimulated with collagen (80 µg/ml) and proteins were lysed and resolved on 10% polyacrylamide gel SDS-PAGE and blotted using phosphoAkt-serine^{473} antibody. Blocking conditions were varied to optimise antibody conditions (**A**) 10% BSA (**B**) 10% milk protein (**C**) 5% BSA (**D**) 5% milk protein.

4.4.2 Globular adiponectin stimulates Akt phosphorylation in a time and dose-dependent manner

It has been demonstrated by several groups that Akt undergoes phosphorylation in response to platelet activation (Kim et al., 2004, Gilio et al., 2009, Kim et al., 2009); therefore the ability of gAd to induce Akt phosphorylation was investigated. Under non-aggregatory conditions, gAd induced Akt serine^{473} phosphorylation in a time and concentration-dependent manner [Figure 4.8]. Phosphorylation of Akt was observed with a concentration of 20 μg/ml, but was maximal at 100 μg/ml. The phosphorylation response was rapid with changes detected within 60 seconds of gAd (100 μg/ml) stimulation. Maximal Akt phosphorylation levels were observed at 90 seconds stimulation before declining back towards basal levels after 300 seconds which was the longest time tested [Figure 4.8].

We next looked at the phosphorylation kinetics involved in CRP-XL and collagen-stimulated Akt phosphorylation. Both CRP-XL and collagen induced Akt serine^{473} phosphorylation in a time and concentration-dependent manner. Akt phosphorylation induced by collagen was detected with a concentration of 20 μg/ml and maximal phosphorylation was observed with 100 μg/ml stimulation [Figure 4.9]. The increase in Akt phosphorylation was rapid and detected within 60 seconds of collagen (100 μg/ml) stimulation and maximal phosphorylation levels were observed at 90 seconds [Figure 4.9]. In contrast, Akt serine^{473} phosphorylation induced by CRP-XL was observed at a concentration of 5 μg/ml and maximal phosphorylation was achieved with 10 μg/ml stimulation [Figure 4.10]. Akt phosphorylation in response to CRP-XL was similar to collagen and gAd with optimum phosphorylation observed at 90 seconds with CRP-XL (10 μg/ml) stimulation [Figure 4.10]. Similar to that of gAd and collagen stimulated Akt phosphorylation, a rapid decrease in Akt phosphorylation at 300 seconds time point was observed which suggests that Akt serine^{473} phosphorylation is not sustained in activated platelets in response to these agonists [Figure 4.10]. Together, these data show that gAd displays similar

kinetics in Akt serine^{473} phosphorylation with other agonists that activate platelets via a tyrosine kinase mechanism.

To further understand the differences between tyrosine kinase and non-tyrosine kinase activation of Akt, we examined Akt phosphorylation induced by thrombin. Thrombin is a potent platelet activator which signals via G-protein coupled receptors, PAR1 and PAR4. Thrombin increased Akt phosphorylation in a concentration-dependent manner, with maximal Akt phosphorylation induced by 1 U/ml thrombin stimulation [Figure 4.11]. Furthermore, we found increase in maximal Akt phosphorylation by thrombin (1 U/ml) stimulation to be rapid and was detected at 60 second time point. However, a sustained Akt phosphorylation was observed between 90 to 300 seconds in thrombin-stimulated platelets [Figure 4.11]. This data suggests that Akt phosphorylation kinetics vary between tyrosine kinase and non-tyrosine kinase activation of Akt.

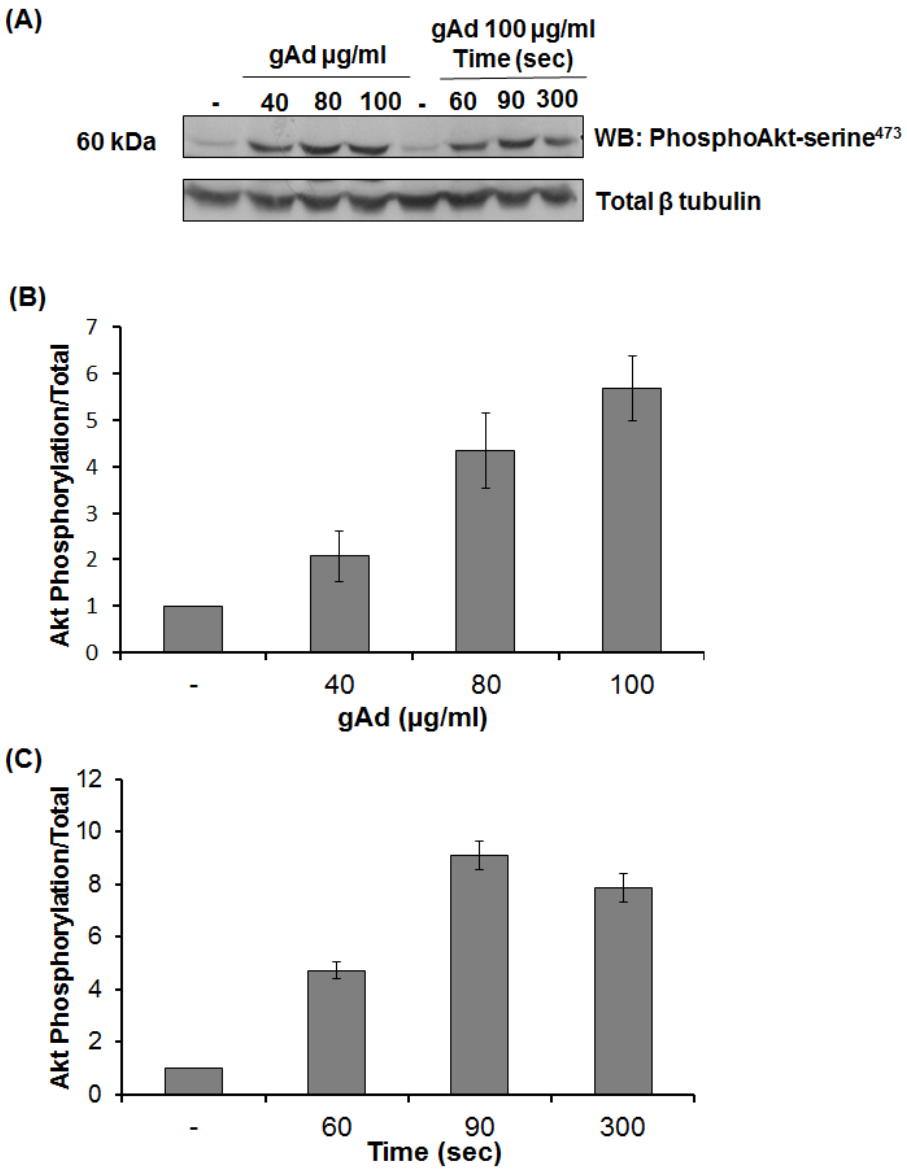


Figure 4.8: Globular adiponectin induces Akt phosphorylation in time and concentration-dependent manner. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with gAd in a time and concentration-dependent manner. Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) and (**C**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as Akt phosphorylation/Total ± SEM of three independent experiments.

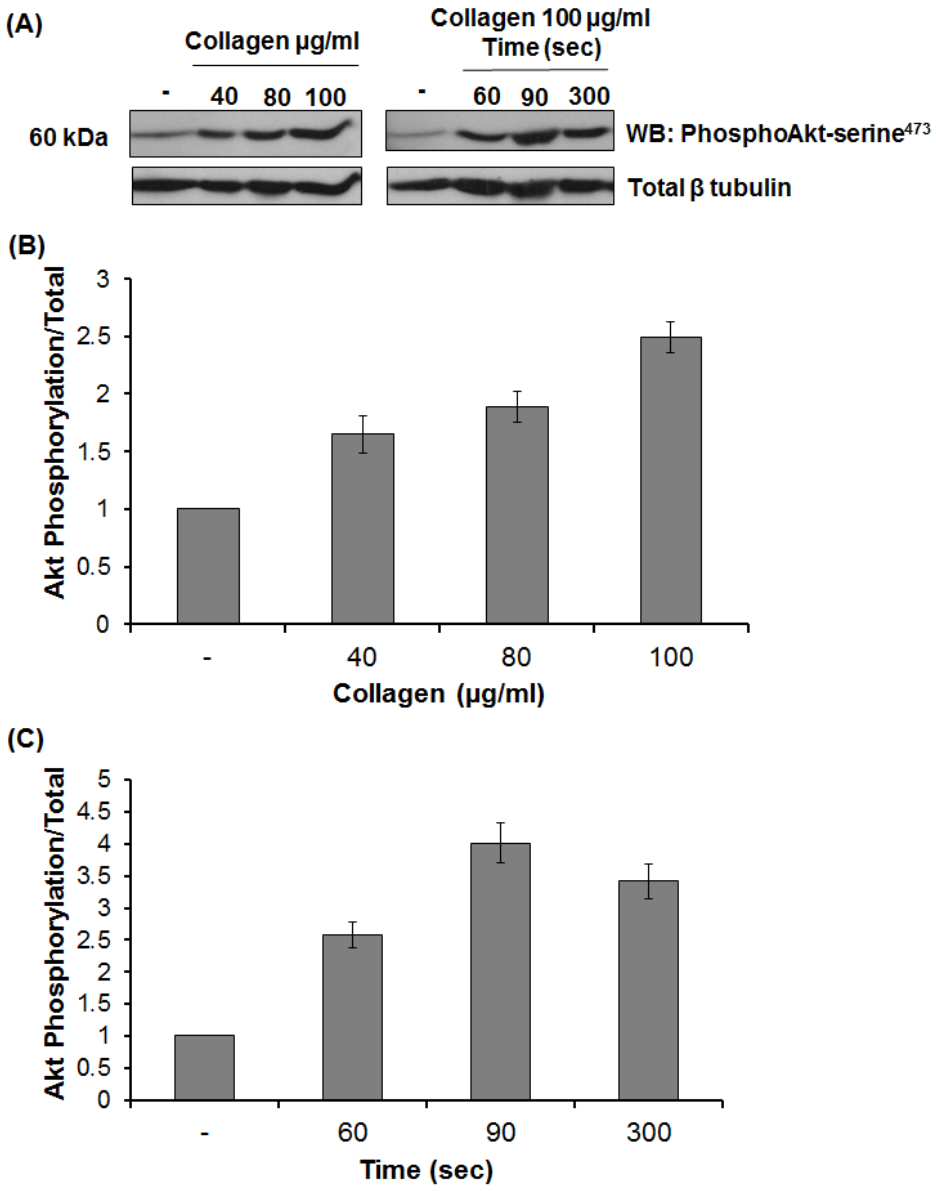


Figure 4.9: Collagen induces Akt phosphorylation in time and concentration-dependent manner. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with collagen in a time and concentration-dependent manner. Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) and (**C**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as Akt phosphorylation/Total ± SEM of three independent experiments.

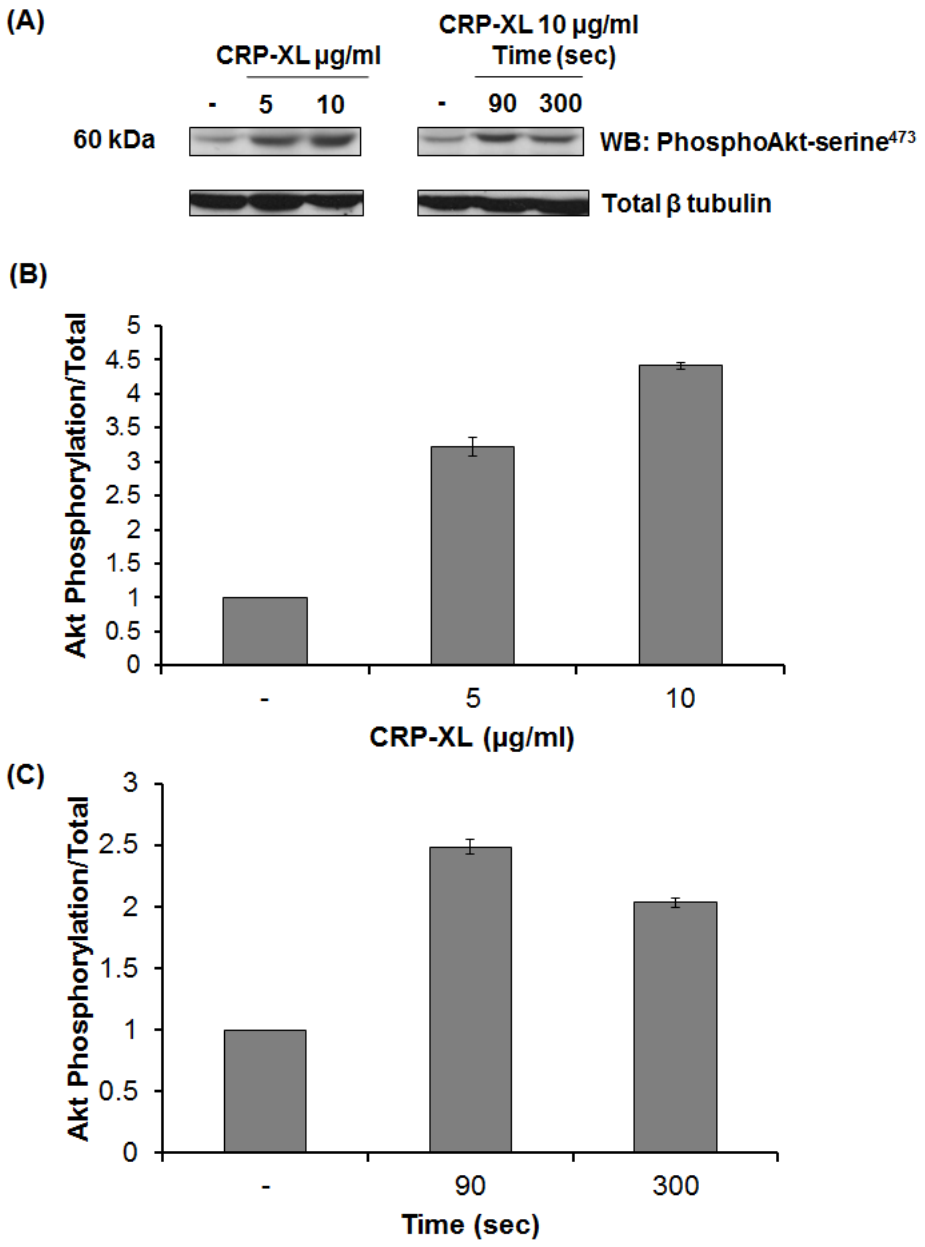


Figure 4.10: CRP-XL induces Akt phosphorylation in time and concentration-dependent manner. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with CRP-XL in a time and concentration-dependent manner. Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) and (**C**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as Akt phosphorylation/Total ± SEM of three independent experiments.

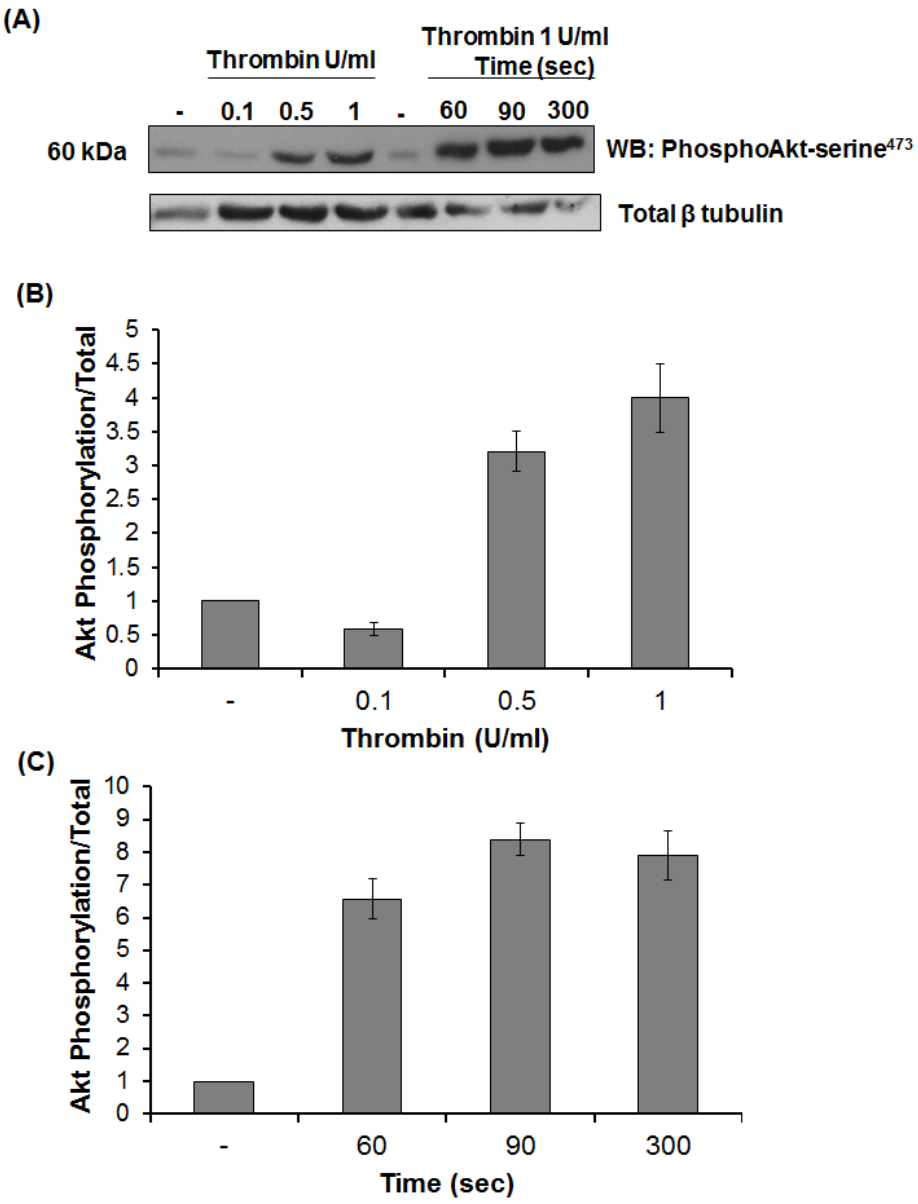


Figure 4.11: Thrombin induces Akt phosphorylation in time and concentration-dependent manner. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with thrombin in a time and concentration-dependent manner. Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) and (**C**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as Akt phosphorylation/Total ± SEM of three independent experiments.

4.4.3 Characterisation of globular adiponectin-induced Akt phosphorylation

Having established that gAd induced the phosphorylation of Akt, we used pharmacological inhibitors to determine the downstream pathways involved in this event. It was important to establish if Akt serine^{473} phosphorylation is dependent on upstream activation of PI3K in gAd-stimulated platelets. This was achieved by stimulating platelets with gAd in the presence of the pan PI3K inhibitor, wortmannin. In these conditions we found that the addition of wortmannin completely inhibited gAd-induced Akt serine^{473} phosphorylation ($p<0.05$) [Figure 4.12]. This data confirms that the activation of Akt is dependent on the upstream activation of PI3K in gAd-stimulated platelets. Furthermore, the treatment of platelets with wortmannin also inhibited collagen and CRP-XL induced Akt serine^{473} phosphorylation and therefore confirming that PI3K is upstream of Akt in GPVI-stimulated platelets [Figure 4.13 and 4.14].

The mobilisation of intracellular Ca^{2+} from the platelet DTS is an important event in the activation of the platelet. We therefore sought to examine the role of intracellular calcium on Akt serine^{473} phosphorylation in gAd-stimulated platelets. Platelets treated with the cell permeable high affinity calcium chelator, BAPTA-AM completely abolished Akt serine^{473} phosphorylation [Figure 4.12]. The chelation of intracellular calcium in collagen or CRP-XL-stimulated platelets also completely abolished Akt serine^{473} phosphorylation [Figure 4.13 and 4.14]. Thus, phosphorylation of Akt under these experimental conditions requires mobilisation of intracellular calcium.

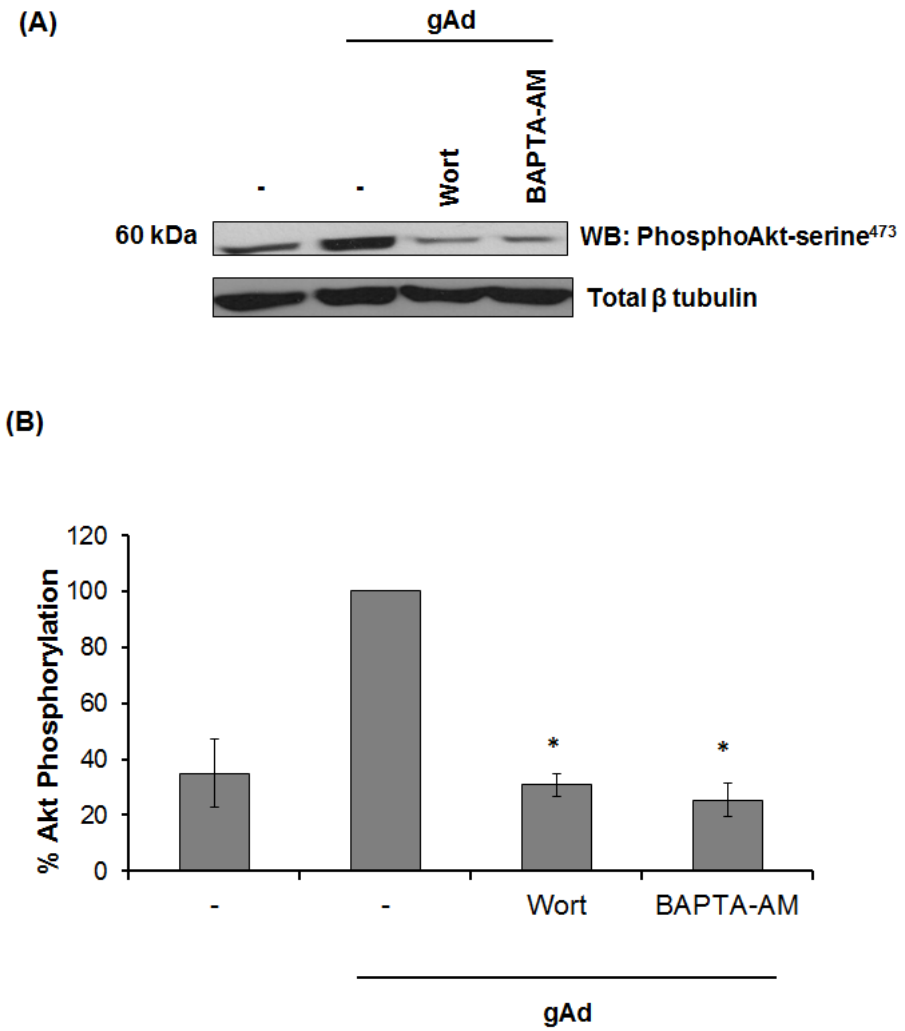


Figure 4.12: Characterisation of Akt phosphorylation in globular adiponectin-stimulated platelets. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with gAd (40 µg/ml) in the presence and absence of wortmannin (100 nM) and BAPTA-AM (10 µM) for 90 seconds. Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with gAd alone to the inhibitors.

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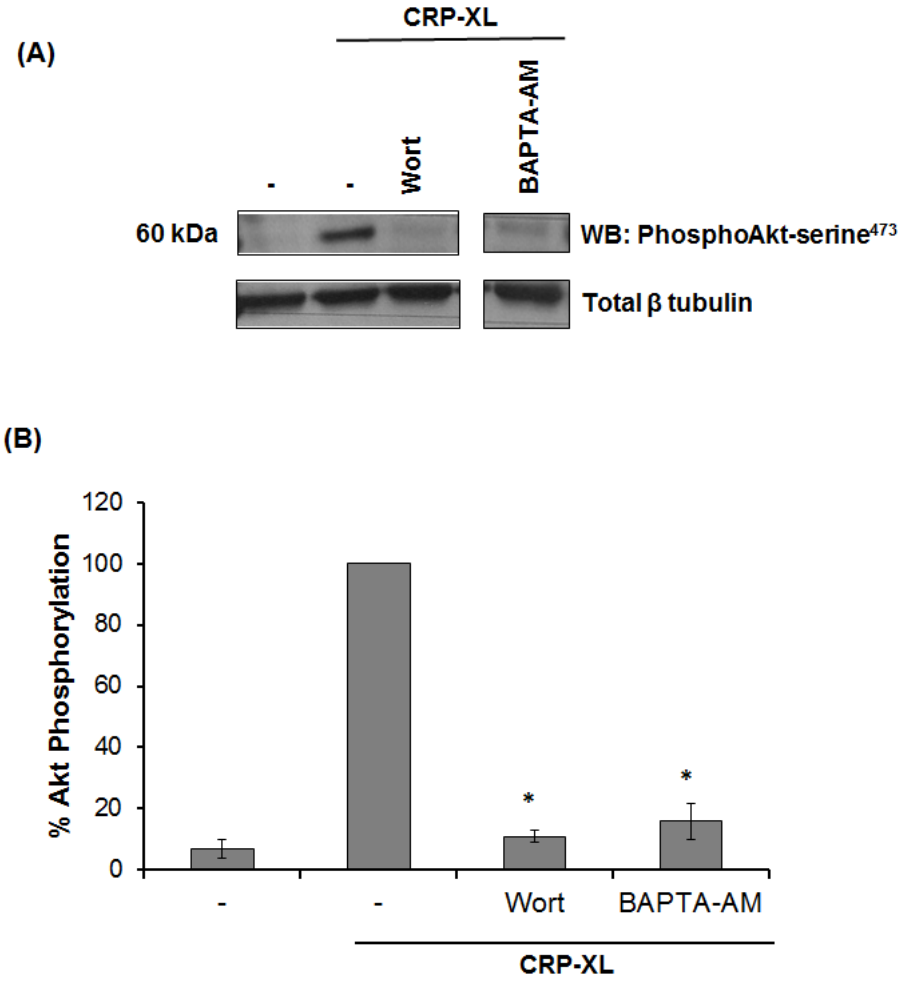

Figure 4.13: Characterisation of Akt phosphorylation in CRP-XL-stimulated platelets. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with CRP-XL (10 µg/ml) in the presence and absence of wortmannin (100 nM) and BAPTA-AM (10 µM) for 90 seconds. Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with CRP-XL alone to the inhibitors.

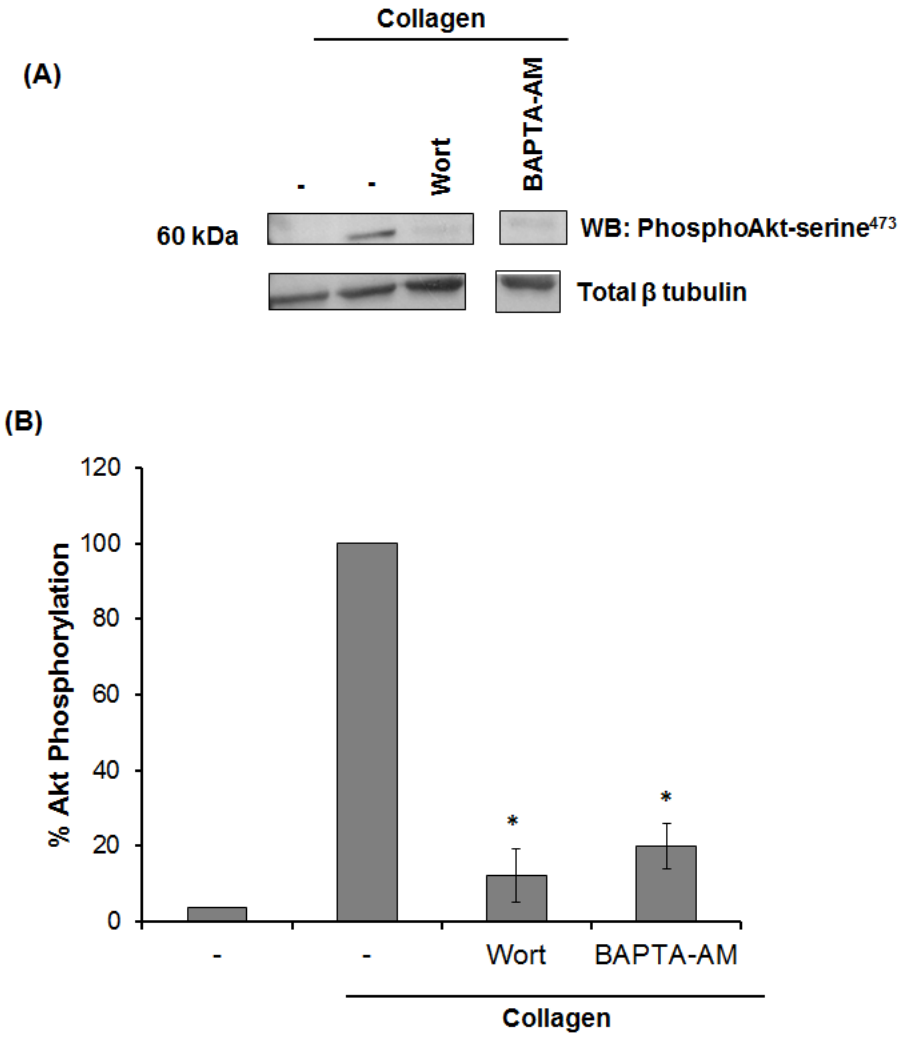

Figure 4.14: Characterisation of Akt phosphorylation in collagen-stimulated platelets. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with collagen (40 µg/ml) in the presence and absence of wortmannin (100 nM) and BAPTA-AM (10 µM) for 90 seconds. Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with collagen alone to the inhibitors.

In the previous chapter we established that gAd-stimulated platelet activation was Src-kinase dependent, therefore the role of these kinases were examined in Akt phosphorylation. Platelets were stimulated with gAd (40 µg/ml) in the presence or absence of the pan Src-kinase inhibitor, PP1 and Akt serine^{473} phosphorylation levels were measured. The presence of PP1 (20 µM) abolished Akt serine^{473} phosphorylation in gAd-stimulated platelets, therefore highlighting a key role for these kinases in Akt activation [Figure 4.15]. Importantly, PP3 (20 µM), the inactive analogue did not affect Akt phosphorylation [Figure 4.15]. Consistent with previous reports (Kim et al., 2009), both CRP-XL and collagen induced Akt serine^{473} phosphorylation were found to be Src-kinase dependent [Figure 4.16 and 4.17]. These data are consistent with a mechanism whereby Akt activation in gAd-stimulated platelets is tyrosine kinase-dependent.

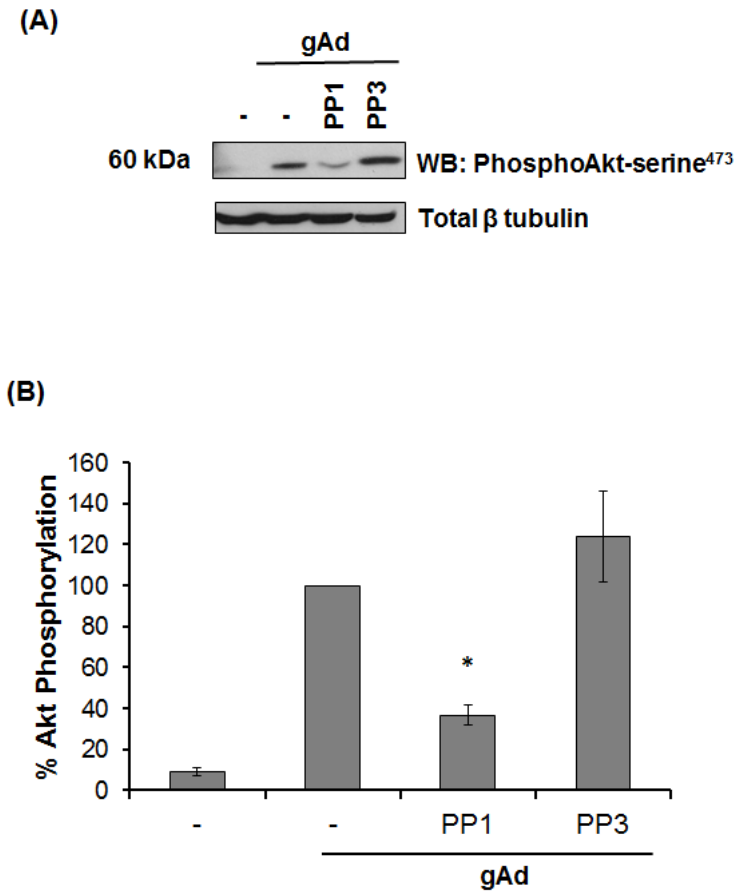

Figure 4.15: Akt serine^{473} phosphorylation is Src-kinase dependent. (**A**) Washed platelets (5x10^{8} platelets/ml) were stimulated with gAd (40 µg/ml) in the presence and absence of PP1 (20 µM) and PP3 (20 µM) for 90 seconds. Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with gAd alone to the inhibitors.

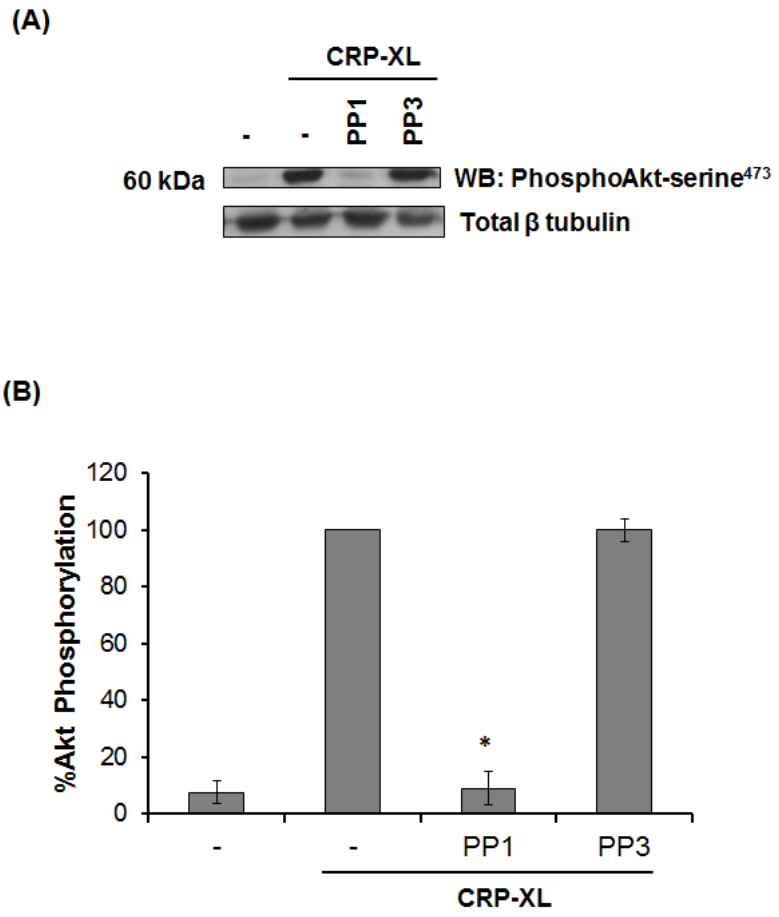

Figure 4.16: Akt serine^{473} phosphorylation is Src-kinase dependent. (**A**) Washed platelets (5x10^{8} platelets/ml) were stimulated with CRP-XL (10 µg/ml) in the presence and absence of PP1 (20 µM) and PP3 (20 µM) for 90 seconds. Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with CRP-XL alone to the inhibitors.

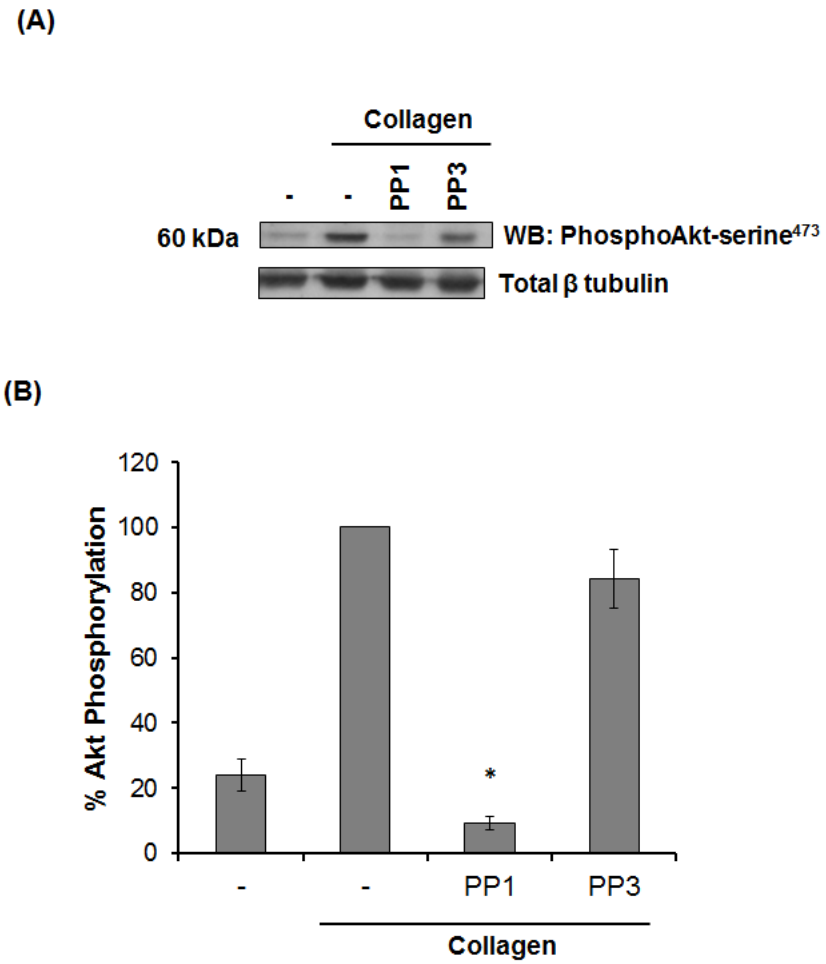

Figure 4.17: Akt serine^{473} phosphorylation is Src-kinase dependent. (**A**) Washed platelets (5x10^{8} platelets/ml) were stimulated with collagen (10 µg/ml) in the presence and absence of PP1 (20 µM) and PP3 (20 µM) for 90 seconds. Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with collagen alone to the inhibitors.

4.4.4 Identification of PI3K isoforms in globular adiponectin-stimulated Akt phosphorylation

Selective PI3K isoform inhibitors were used to understand the roles of individual PI3K isoforms in gAd-stimulated Akt activation. Treatment of gAd-stimulated platelets with TGX-221 (500 nM) ablated Akt serine^{473} phosphorylation in response to gAd ($p<0.05$). In contrast, the presence of both PIK75 (100 nM) and AS242525 (100 nM) led to more modest reductions in Akt serine^{473} phosphorylation [Figure 4.18]. These data suggest that the PI3Kβ isoform plays a significant role in gAd-stimulated Akt phosphorylation, whereas PI3Kα and PI3Kγ play a minor role.

Since gAd-mediated activation of platelets proceeds through tyrosine kinase activation, we compared the roles of PI3K isoforms in CRP-XL and collagen-stimulated platelets. The addition of TGX-221 in CRP-XL-stimulated platelets reduced Akt serine^{473} phosphorylation back to basal levels ($p<0.05$) [Figure 4.19]. In contrast, a partial but significant reduction in Akt serine^{473} phosphorylation was detected with PIK75 ($p<0.05$) [Figure 4.19]. Interestingly, the inhibition of PI3Kγ only had a minor effect on Akt serine^{473} phosphorylation. Similar data was observed with collagen-stimulated platelets [Figure 4.20]. Taken together these data highlight the important roles of PI3K isoforms α and β in tyrosine kinase activation of Akt.

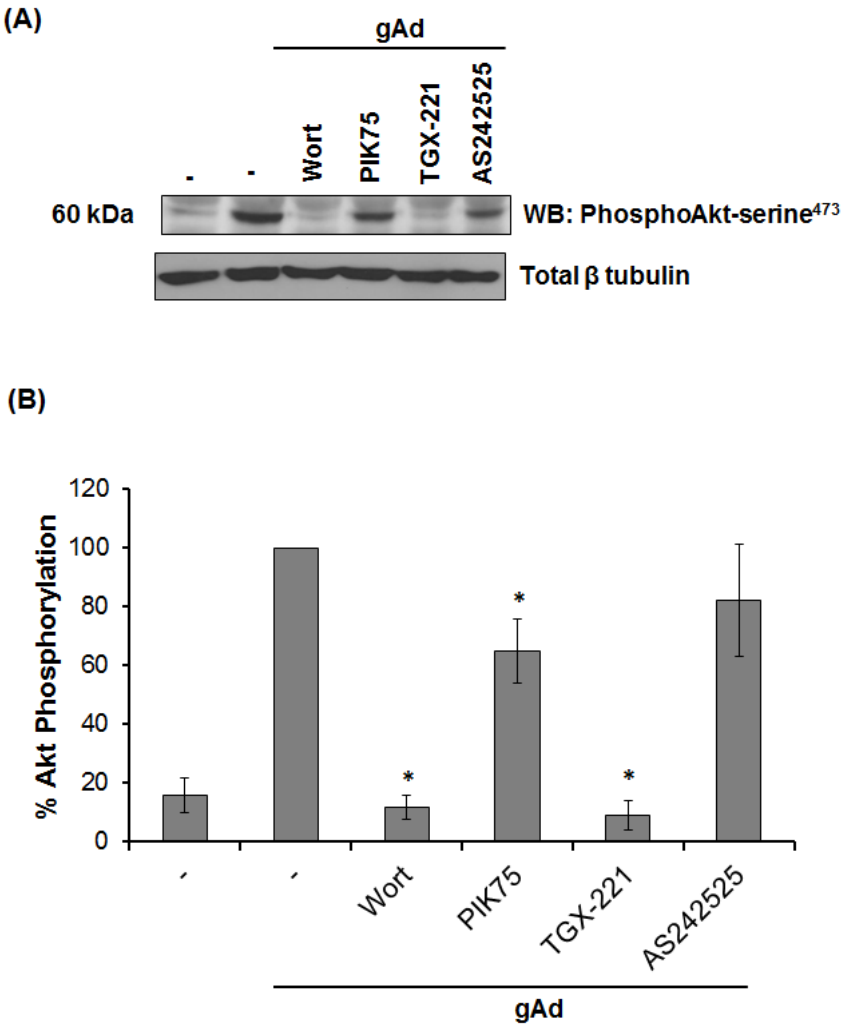

Figure 4.18: The role of PI3K isoforms in globular adiponectin-stimulated Akt serine^{473} phosphorylation. (**A**) Washed platelets (5x10^{8} platelets/ml) were stimulated with gAd (40 µg/ml) in the presence and absence of wortmannin (100 nM), PIK75 (100 nM), TGX-221 (500 nM), AS242525 (100 nM). Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with gAd alone to the inhibitors.

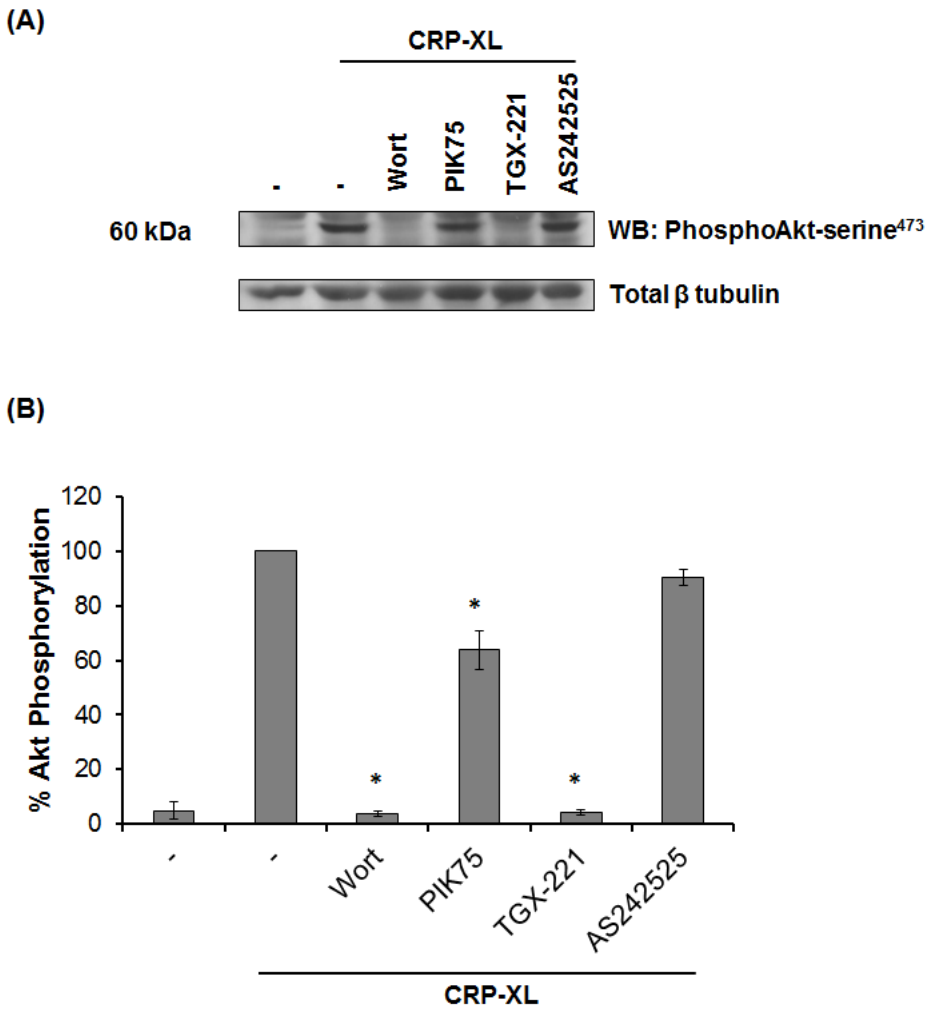

Figure 4.19: The role of PI3K isoforms in CRP-XL-stimulated Akt serine^{473} phosphorylation. (**A**) Washed platelets (5x10^{8} platelets/ml) were stimulated with CRP-XL (10 µg/ml) in the presence and absence of wortmannin (100 nM), PIK75 (100 nM), TGX-221 (500 nM), AS242525 (100 nM). Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with CRP-XL alone to the inhibitors.

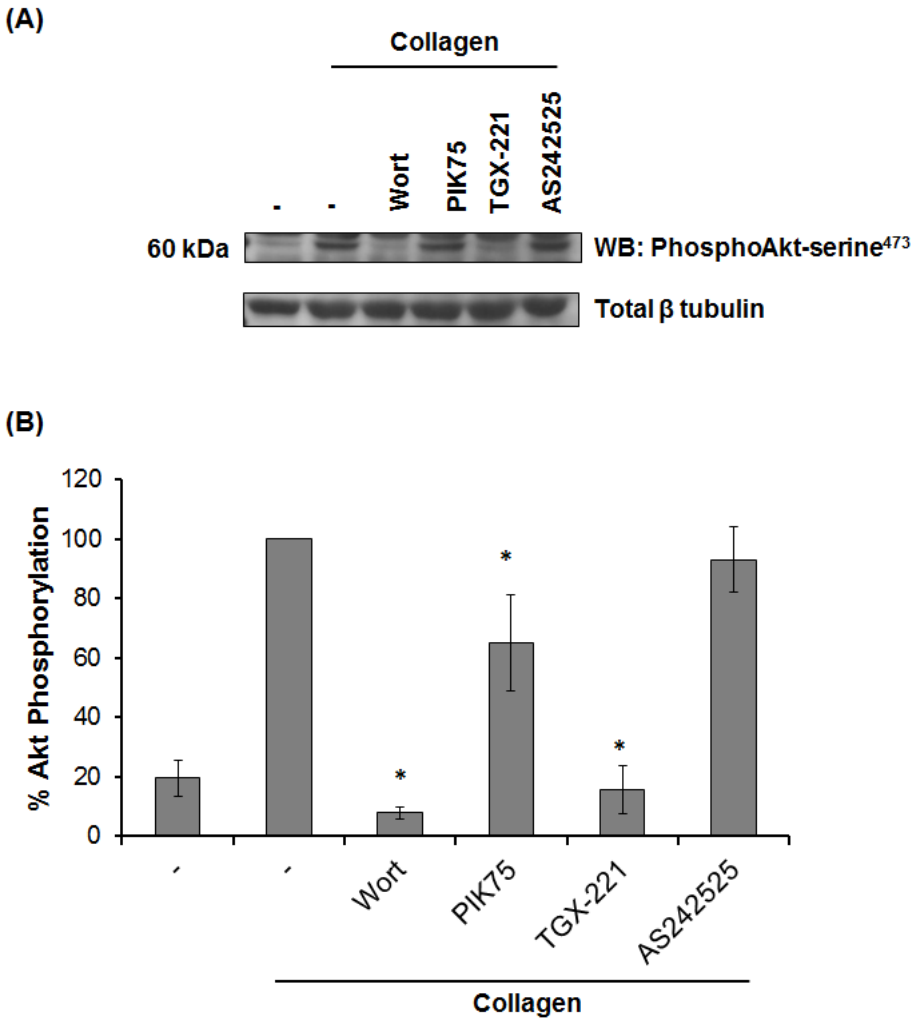

Figure 4.20: The role of PI3K isoforms in collagen-stimulated Akt serine^{473} phosphorylation. (**A**) Washed platelets (5x10^{8} platelets/ml) were stimulated with collagen (10 µg/ml) in the presence and absence of wortmannin (100 nM), PIK75 (100 nM), TGX-221 (500 nM), AS242525 (100 nM). Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with collagen alone to the inhibitors.

4.5 Is secretion required for globular adiponectin-stimulated Akt phosphorylation?

To further understand the signalling events underlying Akt activation, we looked at the role of secondary mediators, ADP and TxA_2 on Akt phosphorylation. To accomplish this we used selective ADP receptor antagonists, A3P5P (300 μM) and MRS2395 (10 μM), which inhibited G_q-coupled $P2Y_1$ and G_i-coupled $P2Y_{12}$ ADP receptors respectively. The cyclo-oxgenase inhibitor, indomethacin (10 μM) was used to inhibit TxA_2 synthesis.

Inhibition of TxA_2 had no effect on gAd-induced Akt phosphorylation, indicating a mechanism that was independent of G_q-coupled $P2Y_1$ and TPα/β signalling [Figure 4.21]. In contrast, combination of ADP receptor antagonists reduced Akt phosphorylation back to basal levels [Figure 4.21]. Further exploration of this observation demonstrated that the $P2Y_{12}$ antagonist MRS2395 significantly reduced Akt serine^{473} phosphorylation, whereas inhibition of $P2Y_1$ only had a minor effect [Figure 4.22]. These results suggest that G_i-coupled $P2Y_{12}$ ADP receptor play an important role in platelet-derived ADP secretion phosphorylation of Akt. A similar dependency of $P2Y_{12}$ stimulation of Akt phosphorylation was observed in both CRP-XL and collagen-stimulated platelets [Figure 4.23 and 4.24]. Taken together, these results suggest that platelet derived TxA_2 plays a minor role compared to ADP which acts through $P2Y_{12}$ receptor to induce Akt serine^{473} phosphorylation.

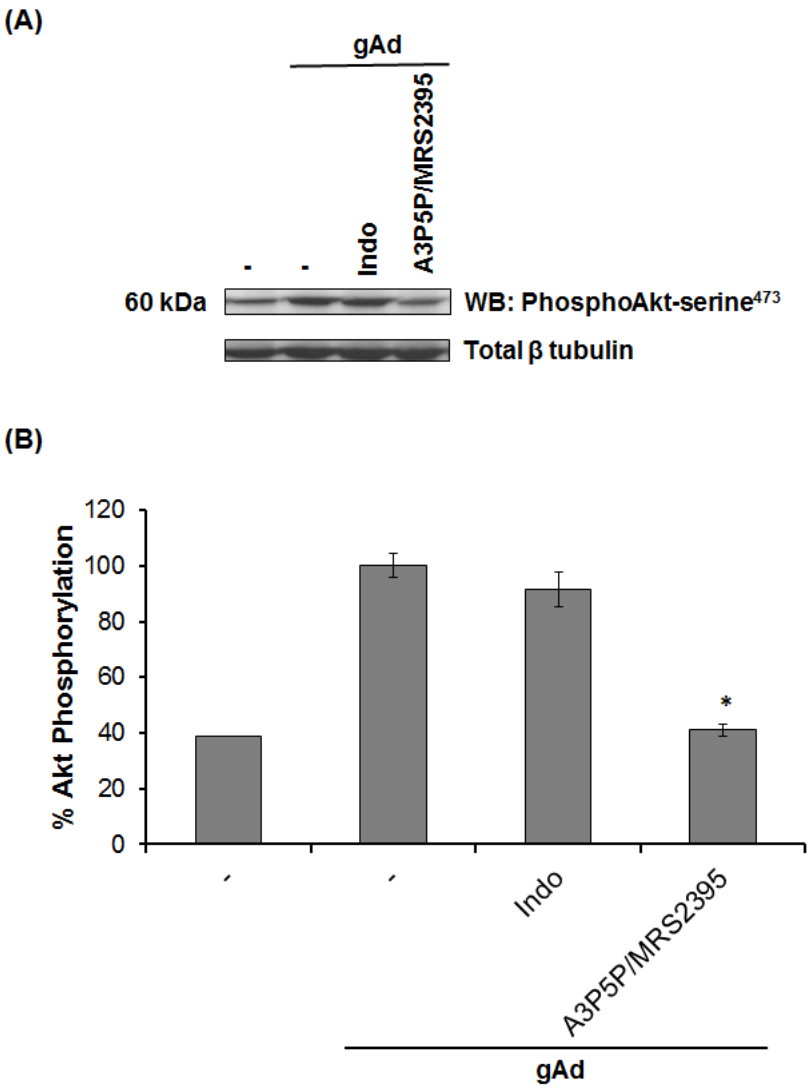

Figure 4.21: The role of secretion on globular adiponectin-stimulated Akt serine^{473} phosphorylation. (**A**) Washed platelets (5x10^{8} platelets/ml) were stimulated with gAd (40 µg/ml) in the presence and absence of indomethacin (10 µM), A3P5P (300 µM) and MRS2395 (10 µM). Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with gAd alone to the inhibitors.

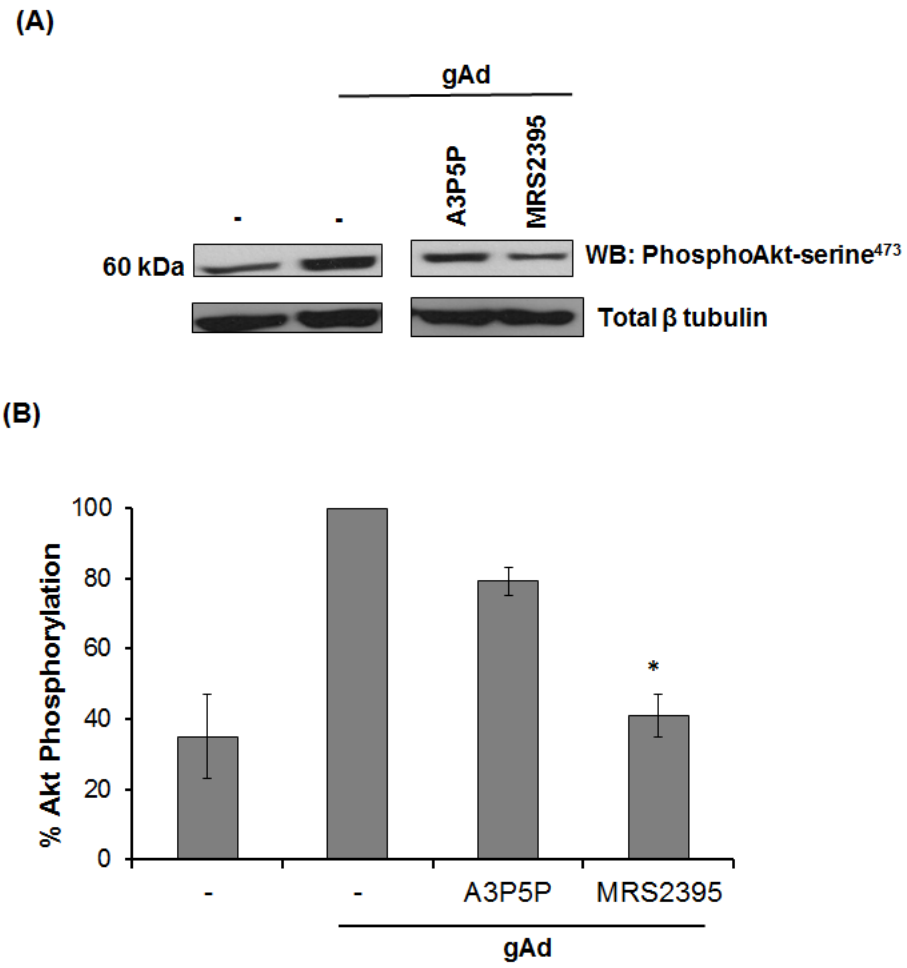

Figure 4.22: The role of ADP secretion on globular adiponectin-stimulated Akt phosphorylation. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with gAd (40 µg/ml) in the presence and absence of ADP receptor antagonists A3P5P (300 µM) and MRS2395 (10 µM). Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with gAd alone to the inhibitors.

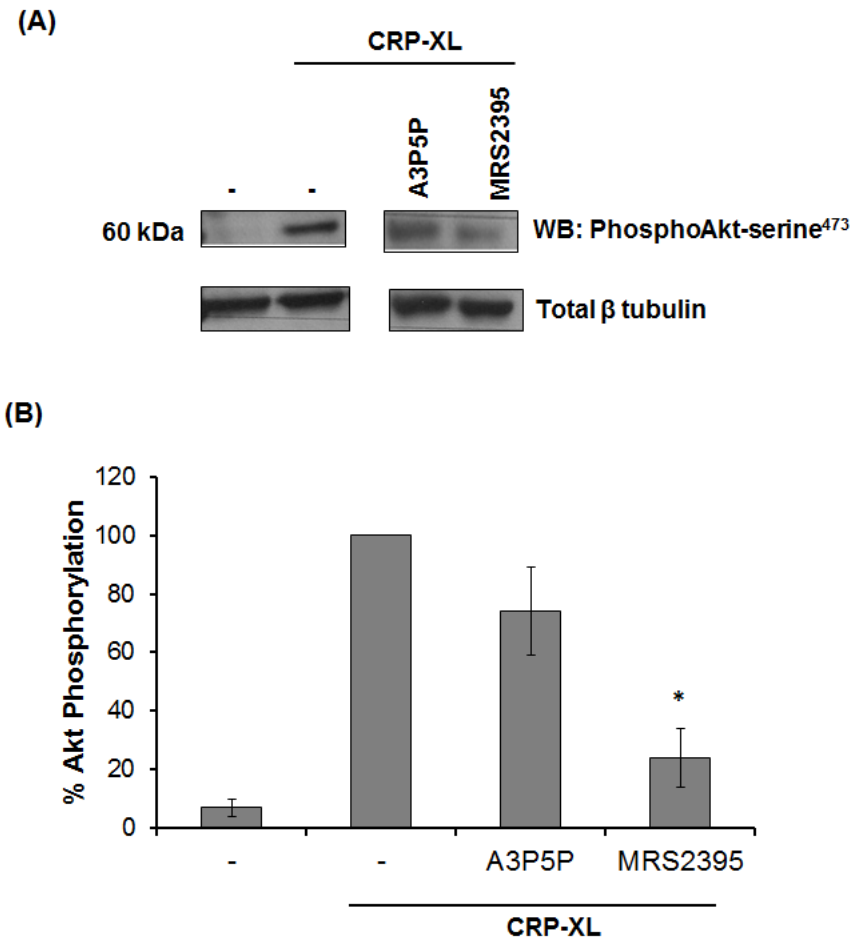

Figure 4.23: The role of ADP secretion on CRP-XL-stimulated Akt phosphorylation. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with CRP-XL (10 µg/ml) in the presence and absence of ADP receptor antagonists, A3P5P (300 µM) and MRS2395 (10 µM). Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with CRP-XL alone to the inhibitors.

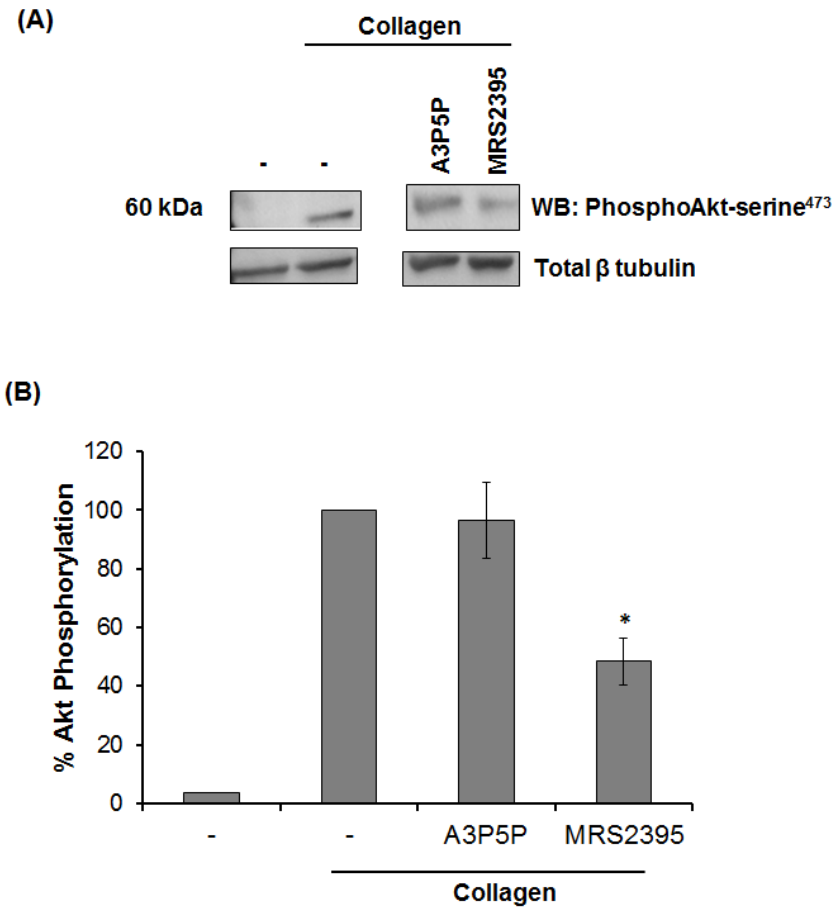

Figure 4.24: The role of ADP secretion on collagen-stimulated Akt phosphorylation. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with collagen (40 µg/ml) in the presence and absence of ADP receptor antagonists, A3P5P (300 µM) and MRS2395 (10 µM). Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with collagen alone to the inhibitors.

4.5.1 The role of PKC on Akt phosphorylation stimulated by globular adiponectin

The secretion of platelet granules is a complex process that is driven at least in part by the family of PKC enzymes (Harper and Poole, 2007). Since our previous data indicated that the secretion of ADP was required for Akt phosphorylation, we examined the role of PKC in this process using a pan PKC inhibitor, Ro 31-8220 (10 μM). Under these conditions of PKC blockade, the ability of gAd to stimulate the phosphorylation of Akt remained undiminished [Figure 4.25]. Interestingly, activation of PMA did lead to the phosphorylation of Akt in platelets indicating that at least one PKC isoform can target Akt activation [Figure 4.25]. Together, these data suggest that gAd-stimulated Akt serine ^{473} phosphorylation is independent of PKC. To confirm that gAd activated the PKC signalling pathway, and Ro 31-8220 inhibited this process, the membranes were reprobed with an antibody that recognises substrates phosphorylation by PKC isoforms. Consistent with our previous data [Figure 3.14], gAd-stimulation increased phosphorylation of a number of PKC substrates, with prominent bands at 50, 55, 60, 70, 110, 120 and 140 kDa [Figure 4.26]. Importantly, the PKC substrate phosphorylation levels of gAd and PMA treated platelets were blocked by the PKC inhibitor Ro 31-8220, thereby confirming the ability of Ro 31-8220 to inhibit PKC [Figure 4.26]. Together, these data suggest that gAd stimulates two distinct pathways that lead to the activation of PKC and phosphorylation of Akt.

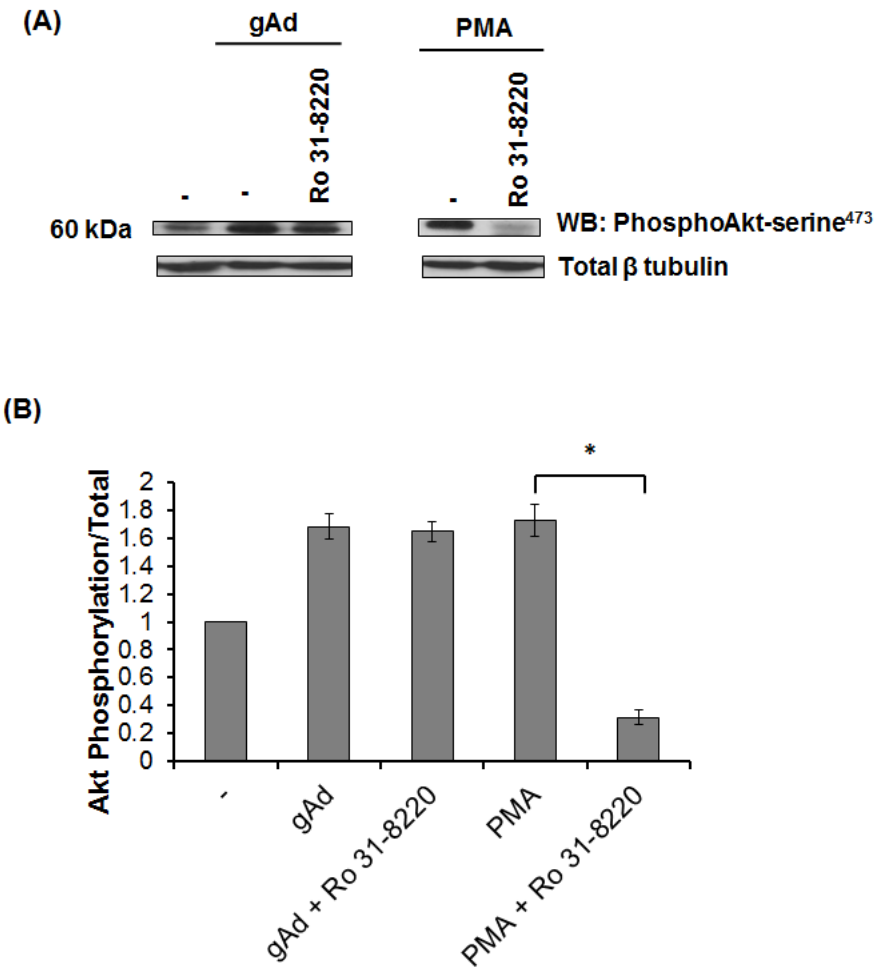

Figure 4.25: The role of PKC on globular adiponectin-stimulated Akt phosphorylation. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with gAd (40 µg/ml) or PMA (100 nM) in the presence and absence of Ro 31-8220 (10 µM). Platelet proteins were separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt Phosphorylation ± SEM of three independent experiments, where * indicates $p<0.05$ compared with PMA alone to the Ro 31-8220.

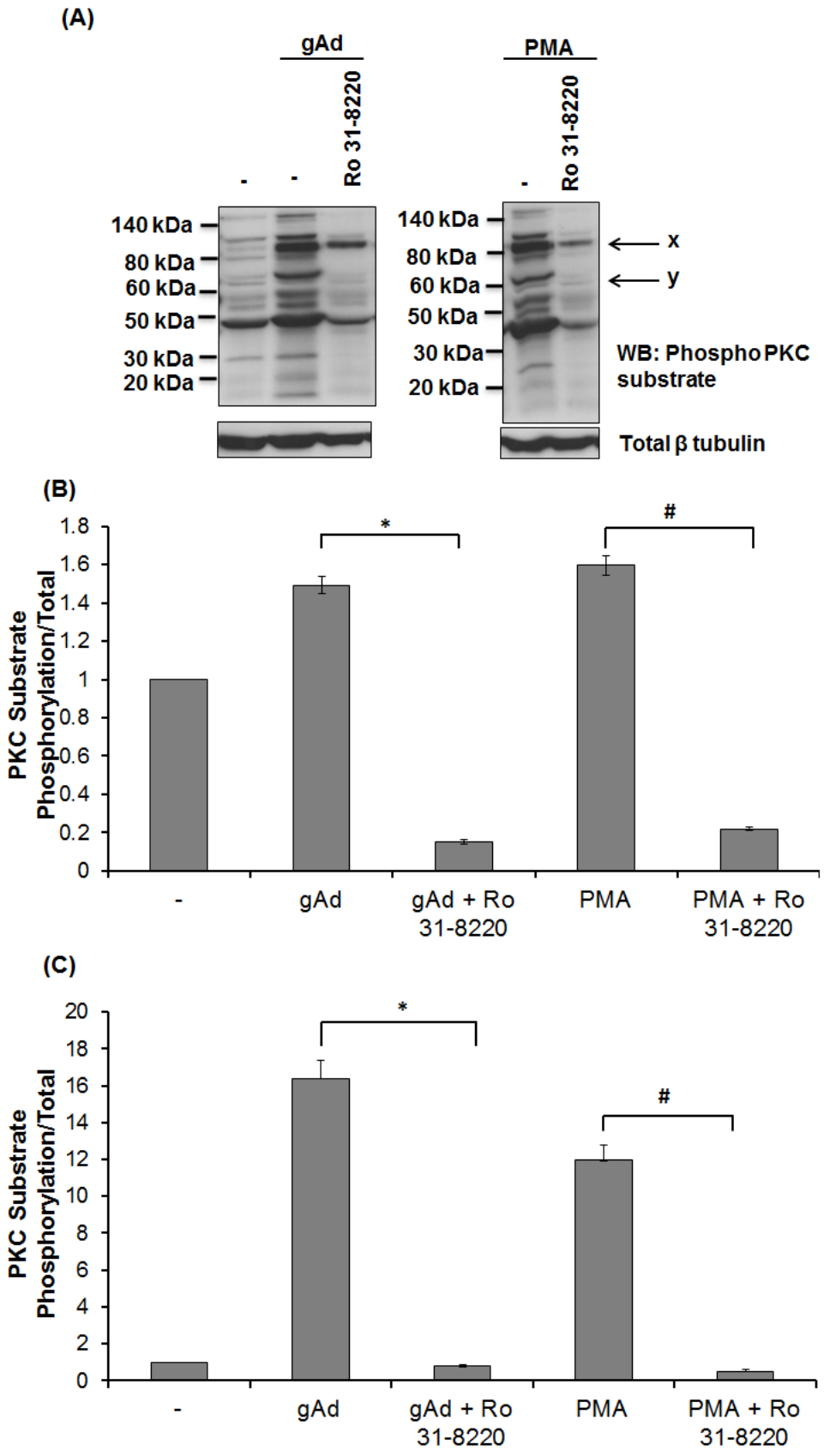

Figure 4.26: The role of PKC on globular adiponectin-stimulated Akt phosphorylation. (**A**) Washed platelets ($5x10^8$ platelets/ml) were stimulated with gAd (40 µg/ml) or PMA (100 nM) in the presence and

absence of Ro 31-8220 (10 μM). Platelets were lysed, separated by SDS-PAGE and blotted with anti-phospho-PKC substrate antibody. (**B**) Densitometric measurement of phospho-PKC substrate at band labelled "x". (**C**) As in (B) except band labelled "y" was selected and expressed as PKC substrate phosphorylation/total ± SEM of three independent experiments, where * indicates $p<0.05$ compared with gAd alone to Ro 31-8220 and # indicates $p<0.05$ compared with PMA alone to Ro 31-8220.

4.6 Identification of signal events underlying PKC-independent secretion in globular adiponectin-stimulated platelets

4.6.1 Synergism between globular adiponectin and G_i signalling in Akt phosphorylation

In chapter 3, we established that gAd can synergise with G_i/G_z mediated signalling to induce aggregation and secretion independently of PKC signalling. Furthermore, the addition of PI3K inhibitor, wortmannin abolished PKC independent secretion. These data suggest a role of PI3K in this process and therefore we measured Akt serine^{473} phosphorylation in these conditions. Platelets were stimulated with gAd in combination with Ro 31-8220 (10 μM), ADP (5 μM) or adrenaline (10 μM) and subjected to immunoblotting. GAd (40 μg/ml) alone stimulated robust Akt serine^{473} phosphorylation which was maintained in the presence of Ro 31-8220 [Figure 4.27]. Importantly, threshold levels of ADP (5 μM) in the presence of $P2Y_1$ receptor antagonist and adrenaline (10 μM) failed to stimulate Akt serine^{473} phosphorylation [Figure 4.28]. However, the stimulation of $P2Y_{12}$ coupled G_i receptor by ADP potentiated Akt serine^{473} phosphorylation compared with Ro 31-8220 alone. Furthermore, this is further evidence that G_i-coupled $P2Y_{12}$ receptor stimulation by ADP is a major pathway responsible for Akt phosphorylation in gAd stimulated platelets.

We next examined the ability of adrenaline to potentiate Akt phosphorylation in conditions of PKC blockade. The stimulation of G_z-coupled α_{2A} adrenoceptor by adrenaline potentiated gAd-mediated Akt serine^{473} phosphorylation in the presence of Ro 31-8220 [Figure 4.27]. Importantly, this potentiation in Akt phosphorylation occurred in the presence of ADP antagonists, indicating that the increased Akt serine^{473} phosphorylation was induced by adrenaline signalling and not because of an indirect effect of secreted ADP. In conditions where platelets were stimulated with ADP, in the presence of A3P5P and adrenaline, we found Akt serine^{473} phosphorylation was significantly potentiated when compared to ADP or adrenaline alone [Figure 4.27]. Taken together, these

data indicate that G_i/G_z signalling can combine with gAd derived signalling to stimulate PI3K downstream phosphorylation of Akt, which may compensate for the loss of PKC activity.

It is established that ADP can potentiate GPVI-collagen-stimulated aggregation and therefore indicate a synergism between tyrosine kinase-linked and G-protein coupled receptor-linked pathways (Atkinson et al., 2001, Dangelmaier et al., 2001). It was important to determine the role of PI3K in this process. Since the data suggested that gAd and G_i/G_z signalling can synergise to compensate for the loss of PKC signalling, we repeated these experiments with a GPVI specific agonist, CRP-XL. We found that CRP-XL induced Akt serine^{473} phosphorylation and was maintained in the presence of Ro 31-8220 [Figure 4.28]. The addition of ADP under conditions of PKC inhibition, acting through G_i-coupled $P2Y_{12}$ receptor potentiated Akt serine^{473} phosphorylation compared to CRP-XL alone [Figure 4.28]. The addition of adrenaline under these same conditions of PKC blockade also potentiated Akt serine^{473} phosphorylation. Importantly, stimulation of G_i-coupled $P2Y_{12}$ receptor and G_z-coupled α_{2A} adrenoceptor with ADP and adrenaline respectively even further potentiated Akt serine^{473} phosphorylation. Taken together, these data suggest an important role for PI3K in the synergism between tyrosine kinase-linked and G-protein coupled receptor-linked pathways.

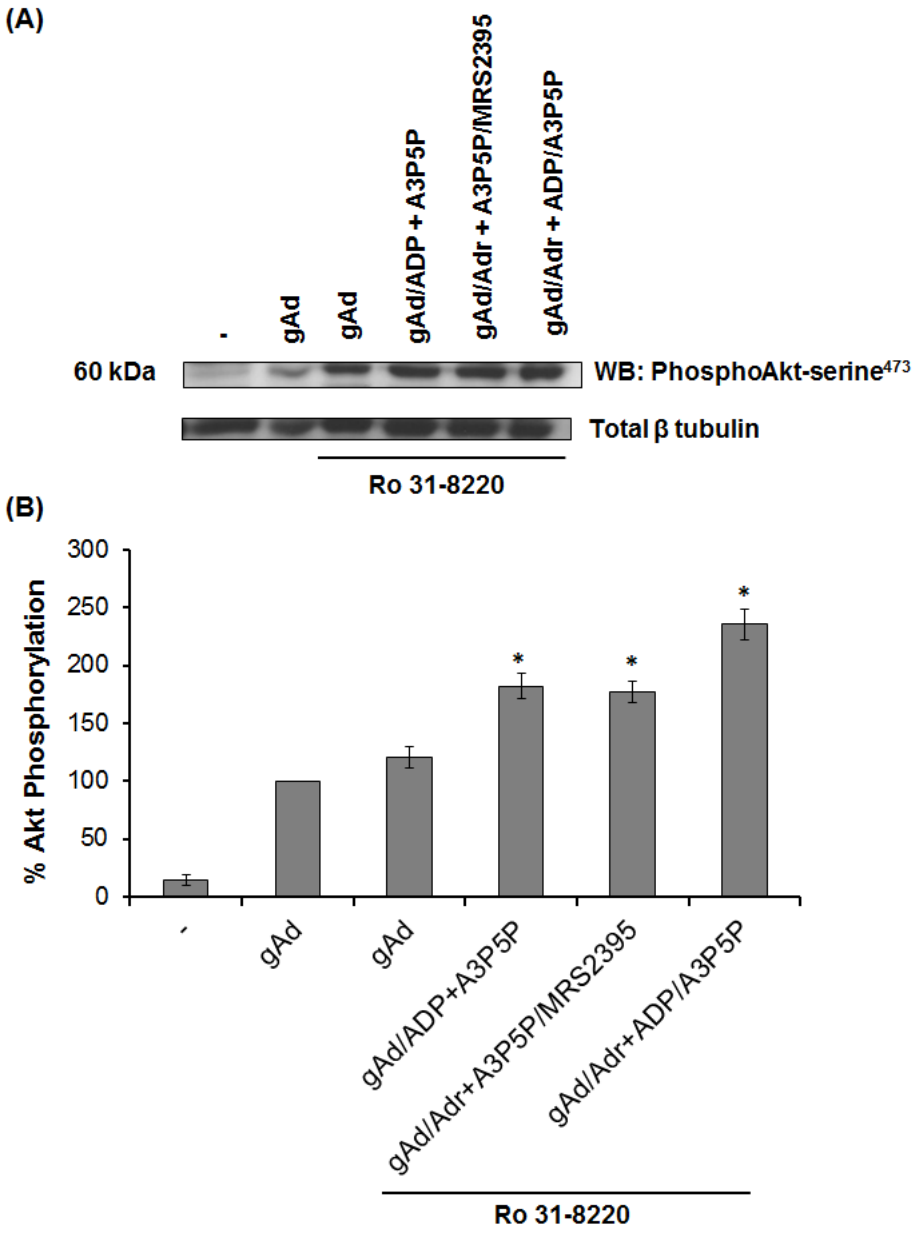

Figure 4.27: Akt serine^{473} phosphorylation is potentiated with G_i/G_z signalling in globular adiponectin-stimulated platelets. (**A**) Washed platelets (5x10^8 platelets/ml) were pre-incubated with Ro 31-8220 (10 µM) and then stimulated with gAd (40 µg/ml) alone or in combination with ADP (5 µM), or adrenaline (10 µM). In some cases platelets, were pretreated with A3P5P (300 µM) or MRS2395 (10 µM). Platelet proteins were lysed and separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of

three independent experiments, where * indicates $p<0.05$ compared with gAd and Ro 31-8220 to the inhibitors.

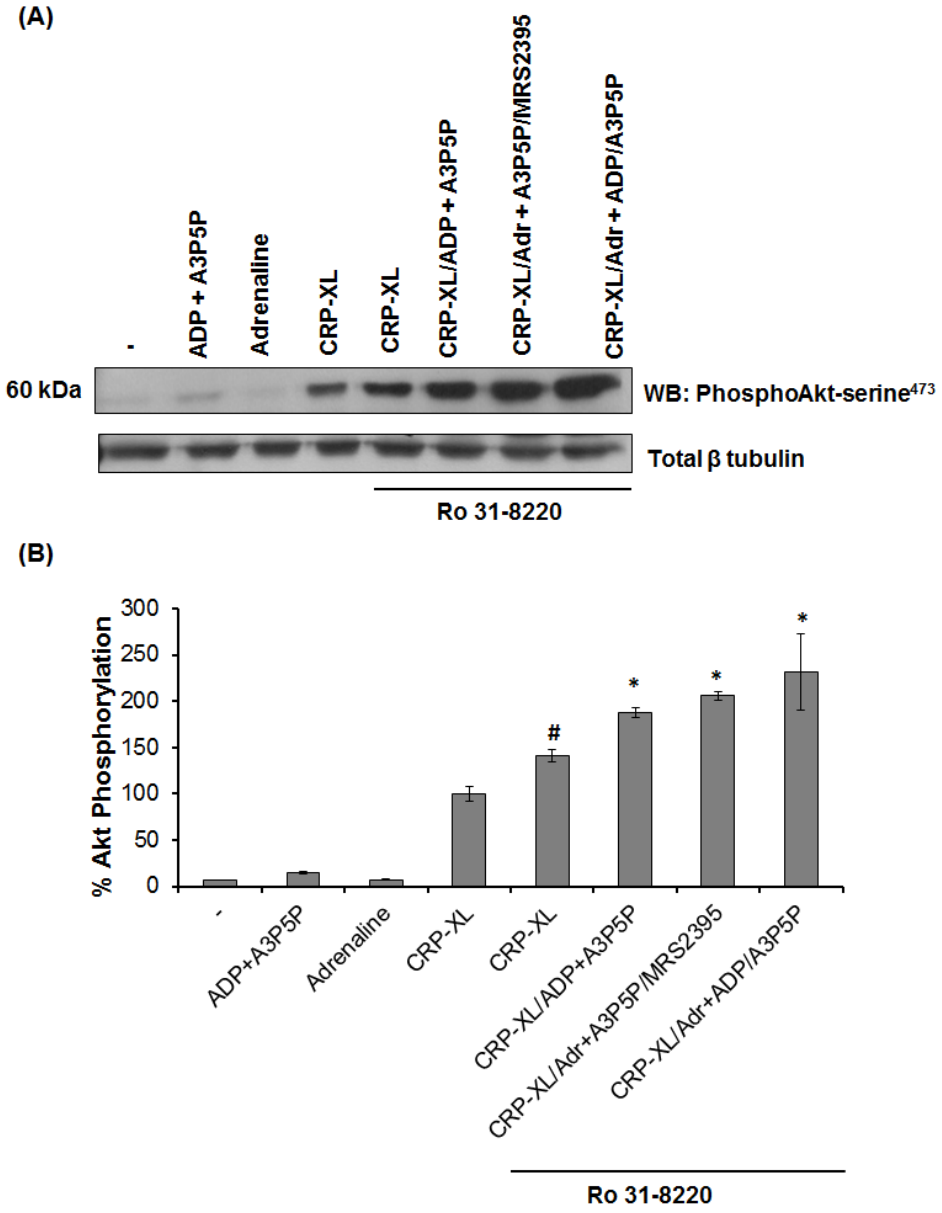

Figure 4.28: Akt serine^{473} phosphorylation is potentiated with G_i/G_z signalling in CRP-XL-stimulated platelets. (**A**) Washed platelets (5x10^8 platelets/ml) were pre-incubated with Ro 31-8220 (10 µM) and then stimulated with CRP-XL (10 µg/ml) alone or in combination with ADP (5 µM), or adrenaline (10 µM). In some cases platelets were pre-treated with A3P5P (300 µM) or MRS2395 (10 µM). Platelet proteins were lysed and separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent experiments, where # indicates $p<0.05$ compared with CRP-XL

alone to Ro 31-8220 and * indicates $p<0.05$ compared with CRP-XL and Ro 31-8220 to the inhibitors.

4.6.2 The role of PI3K isoforms in synergism between globular adiponectin and G_i/G_z signalling in Akt phosphorylation

Having established that Akt phosphorylation is potentiated in the synergy between gAd and G_i/G_z signalling, we used selective PI3K isoform inhibitors to determine the contribution of these isoforms in this process. This was achieved by measuring the combined ADP and adrenaline induced potentiation of Akt serine^{473} phosphorylation in pre-treated Ro 31-9220 (10 μM) platelets, in absence and presence of selective PI3K isoform inhibitors.

Under these conditions, the addition of pan PI3K inhibitor wortmannin abolished the potentiation of Akt phosphorylation [Figure 4.29]. This data confirms our previous findings that PI3K plays an integral role in this pathway. Selective PI3K inhibitors were then used to identify which isoform were involved in this process. The addition of PI3Kβ inhibitor TGX-221 abolished Akt phosphorylation ($p<0.05$) [Figure 4.29]. Furthermore, the addition of PI3Kα inhibitor PIK75 partially reduced Akt serine^{473} phosphorylation ($p<0.05$) [Figure 4.29]. However, inhibition of PI3Kγ by AS242525 failed to prevent the potentiation of Akt phosphorylation [Figure 4.29]. Similar data was obtained with CRP-XL-stimulated platelets [Figure 4.30]. These data implicate a key role of PI3Kβ and PI3Kα isoforms in gAd and G_i/G_z mediated signalling to overcome the loss of PKC activity.

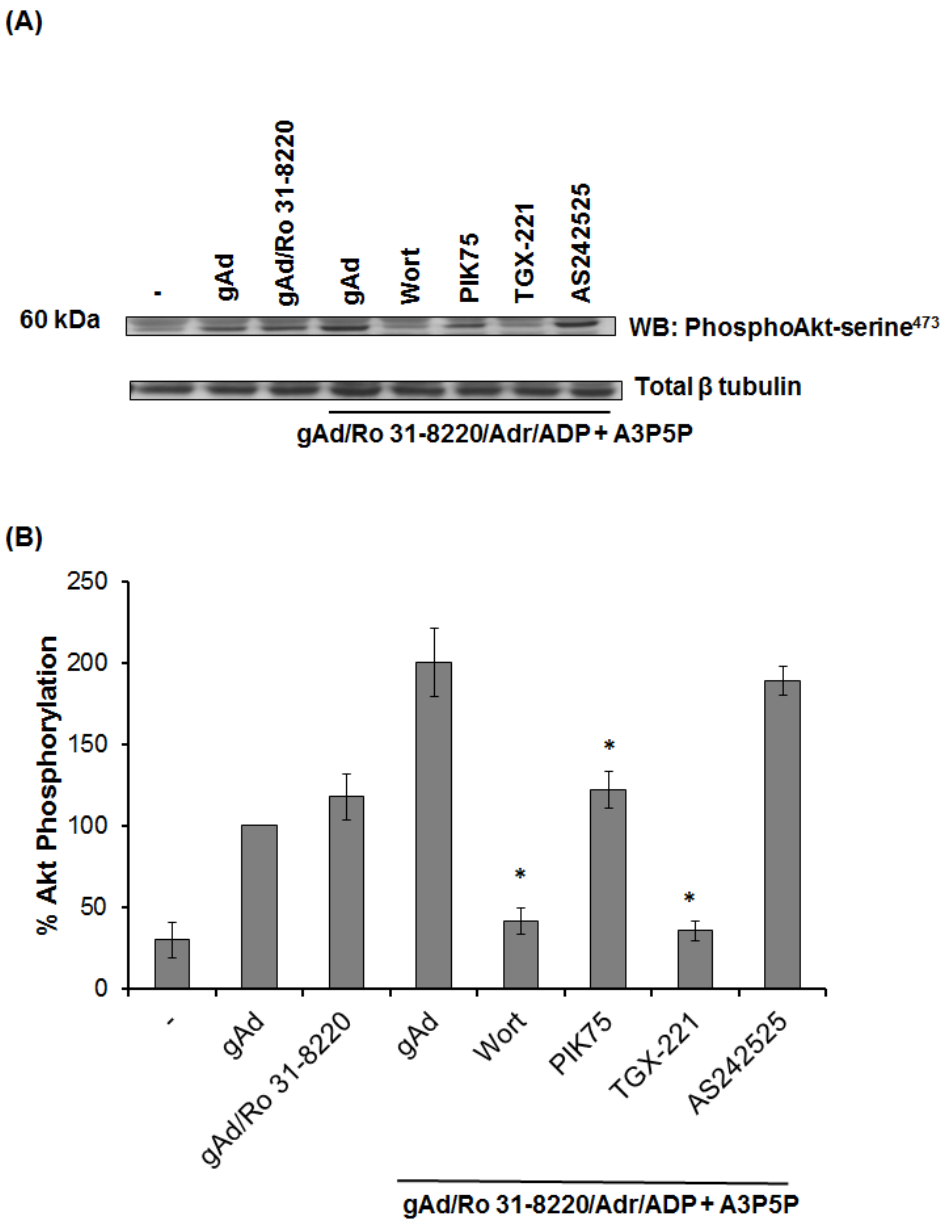

Figure 4.29: The role of PI3K isoforms in G_i/G_z potentiation of Akt serine^{473} phosphorylation in globular adiponectin-stimulated platelets. (**A**) Washed platelets (5x10^8 platelets/ml) were pre-incubated with Ro 31-8220 (10 µM) and then stimulated with gAd (40 µg/ml) in combination with ADP (5 µM), adrenaline (10 µM) and A3P5P (300 µM) in the presence of PI3K inhibitors; wortmannin (100 nM), PIK75 (100 nM), TGX-221 (500 nM) and AS242525 (100 nM) Platelet proteins were lysed and separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent

experiments, where * indicates $p<0.05$ compared with gAd/Ro 31-8220/Adrenaline/ADP + A3P5P to the PI3K inhibitors.

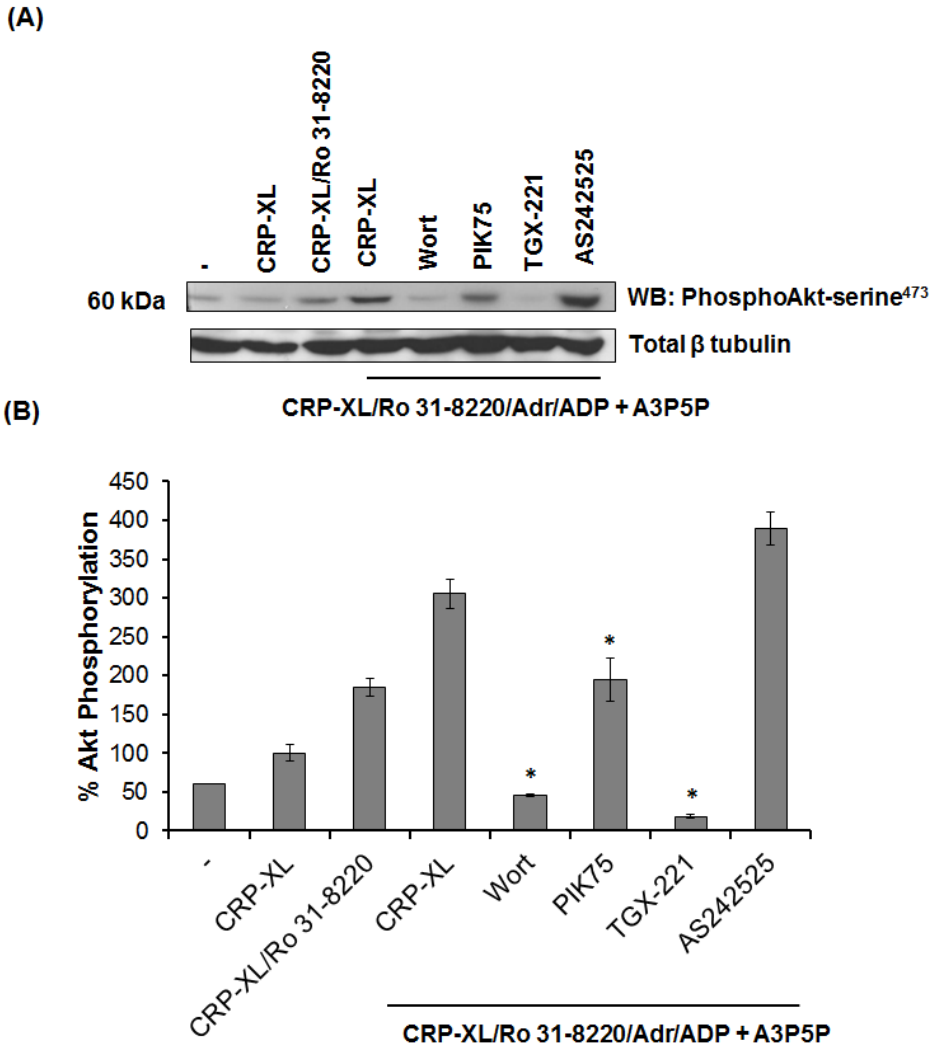

Figure 4.30: The role of PI3K isoforms in G_i/G_z potentiation of Akt serine^{473} phosphorylation in CRP-XL-stimulated platelets. (**A**) Washed platelets (5x10^8 platelets/ml) were pre-incubated with Ro 31-8220 (10 µM) and then stimulated with CRP-XL (10 µg/ml) in combination with ADP (5 µM), adrenaline (10 µM) and A3P5P (300 µM) in the presence of PI3K inhibitors; wortmannin (100 nM), PIK75 (100 nM), TGX-221 (500 nM) and AS242525 (100 nM) Platelet proteins were lysed and separated by SDS-PAGE and blotted for phosphoAkt-serine^{473}, followed by re-probing for β-tubulin. (**B**) Densitometric measurement of phosphoAkt-serine^{473} and expressed as %Akt phosphorylation ± SEM of three independent

experiments, where * indicates $p<0.05$ compared with CRP-XL/Ro 31-8220/Adrenaline/ADP + A3P5P to the PI3K inhibitors.

4.7 Discussion

In this chapter the contribution of PI3K to gAd-stimulated platelet aggregation was studied. We had already determined that gAd stimulates platelet aggregation in two distinct phases. The first phase is mediated by direct activation via a tyrosine kinase-dependent signalling cascade. The second phase is dependent upon the release of ADP and TxA_2 which potentiate platelet aggregation. However, the role of PI3K in gAd-stimulated aggregation is not known. Here, we found that gAd stimulated aggregation through pathways requiring PI3K activation, which was independent of tyrosine phosphorylation levels. Further experiments with selective PI3K isoform inhibitors revealed that class IA PI3K isoforms α and β are activated downstream of gAd activation and contribute to platelet aggregation and secretion. Riba et al., had shown previously that platelet stimulation by gAd proceeds through activation of Syk, LAT and PLCγ2 (Riba et al., 2008). These data suggest that similar to GPVI signalling, PI3K activation may be regulated by tyrosine kinases. The exact mechanism is not known, but may involve the formation of a signalosome containing LAT, PI3K and PLCγ2. It is now well established that GPVI signalling involves the activation of PI3K and PLCγ2 which interact with other adapter proteins such as LAT to form a signalosome, leading to integrin $\alpha_{IIb}\beta_3$ activation (Songyang et al., 1994, Nieswandt and Watson, 2003). It is possible that a similar mechanism is used in gAd-stimulated platelets. Furthermore, the assembly of a signalosome containing PI3K and PLCγ2 allows a direct association with their substrates. For example, increased PIP_2 production by PI3K can directly interact with PLCγ2 (Pasquet et al., 1999, Martin et al., 2010). In fact, PLCγ2 activity can be reduced by up to 80% by the inhibition of PI3K in CRP-XL-stimulated platelets (Pasquet et al., 1999). This suggests that the inhibitory effects of wortmannin are mediated through pathways that are independent of tyrosine phosphorylation.

To understand the signalling events underlying PI3K activation, we examined Akt serine^{473} phosphorylation levels as a marker of PI3K activation (Cantley, 2002). Akt is a serine-threonine kinase and its activity

can be directly regulated by the production of phosphatidylinositol. We showed for the first time that gAd-stimulated Akt serine^{473} phosphorylation in a time and concentration-dependent manner. Consistent with data from other platelet agonists, Akt serine^{473} phosphorylation in response to gAd was completely abolished by PI3K inhibition. Further investigation of the potential PI3K isoforms responsible suggested that both α and β may play a role. However, from the current data it is predicted that PI3Kβ isoform was quantitatively more important since inhibition of this isoform fully blocked Akt serine^{473} phosphorylation, while inhibition of the α-isoform caused only a partial inhibition. Furthermore, Akt serine^{473} phosphorylation was dependent on pathways involving Src-kinase family isoforms and required calcium mobilisation. This is consistent with a mechanism whereby gAd-stimulates Akt phosphorylation through PI3K and Src-kinase dependent signalling pathways that require calcium. Importantly, this pathway involving Akt phosphorylation through PI3K and Src-kinase signalling can be found in the literature regarding platelet activation involving collagen and GPVI receptor (Pasquet et al., 1999, Nieswandt and Watson, 2003).

The release of platelet secondary mediators such as ADP and TxA_2 are required for maximal gAd-stimulated aggregation. Inhibition of TxA_2 signalling had no effect on Akt serine^{473} phosphorylation. However, antagonism of ADP receptors was able to ablate Akt serine^{473} phosphorylation levels, suggesting a key role of this agonist. Selective antagonism of ADP receptors revealed that Akt serine^{473} phosphorylation was dependent on G_i-coupled $P2Y_{12}$ signalling. These data suggest that G_i signalling is required for maximal PI3K activity when platelets are stimulated by gAd. Since the release of ADP from platelet dense-granules is driven by pathways that require PKC, we examined the role of this kinase in Akt activation. Interestingly, the inhibition of PKC did not influence Akt serine^{473} phosphorylation levels, suggesting that Akt serine^{473} phosphorylation is independent of PKC. It has been reported that Akt serine^{473} is phosphorylated by PKCβ2 isoform in mast cells (Kawakami et

al., 2004). However, platelets only express the PKCβ1 isoform and the role of this isoform in Akt phosphorylation in platelets is unclear.

Platelets contain redundant pathways that can compensate for the loss of platelet signalling pathways. For example, the loss of the collagen GPVI receptor can be compensated by other platelet activation pathways and therefore only display a mild bleeding phenotype (Arai et al., 1995). However, some of these compensatory systems can work with existing systems to promote platelet activation and thrombus formation. The current data suggests such a system that involves the synergy between gAd and G_i/G_z signalling pathways to promote platelet aggregation and secretion independent of PKC. In chapter 3, evidence was presented to demonstrate the potential existence of a PKC-independent pathway for dense-granule secretion. Here, it was found that the addition of a PI3K inhibitor abolished PKC-independent secretion suggesting a role of PI3K in this process. Three distinct pieces of evidence are present to support this;

1. GAd-stimulated Akt serine^{473} phosphorylation remained under conditions of PKC inhibition.

2. The addition of either ADP or adrenaline, alone or in combination, with gAd potentiated Akt serine^{473} phosphorylation under conditions of PKC inhibition. An effect that was blocked by the PI3K inhibitor, wortmannin.

3. The effects of wortmannin could be repeated with structurally distinct isoform specific inhibitors of PI3K.

The G_i-coupled $P2Y_{12}$ receptor has been reported to be linked to PI3K isoforms β and γ. Selective PI3K inhibitors revealed that only PI3Kβ isoform and to a lesser extent PI3Kα isoform are involved in the potentiation. This is consistent with reports that PI3Kβ isoform is major contributor to platelet activation downstream of $P2Y_{12}$ (Jackson et al., 2005, Schoenwaelder et al., 2007, Canobbio et al., 2009). The exact importance of PI3Kβ and γ isoforms downstream of $P2Y_{12}$ is controversial. The

activation of G_i-coupled $P2Y_{12}$ by ADP is shown only to involve PI3Kβ activation and not PI3Kγ (Garcia et al., 2010, Kim and Kunapuli, 2011). However, some studies with PI3Kγ-deficient mice show reduced responses to ADP, but responses to other agonists were normal (Jackson et al., 2005, Schoenwaelder et al., 2007). These studies suggest that PI3Kγ activation is not required for maximal platelet activation. However, the role of class IA PI3Kα isoform in gAd-stimulated platelet activation is less clear. It has been reported that PI3Kα along with PI3Kβ play an important role in platelet activation downstream of the collagen receptor GPVI in the absence of any secondary mediators (Watanabe et al., 2003, Kim et al., 2009). It has been previously suggested that gAd may activate platelets via the collagen receptor GPVI (Riba et al., 2008). Therefore, it is possible that PI3Kα isoform maybe activated downstream of GPVI activation.

In this study we have identified PI3Kβ as a critical component in the potentiation of platelet responses to gAd via G_i/G_z signalling. It has been shown that PI3Kβ plays an important role in G-protein coupled receptors and GPVI activation (Rittenhouse, 1996, Canobbio et al., 2009, Kim et al., 2009). Platelet responses to ADP downstream of $P2Y_{12}$ have been shown to have an absolute requirement for PI3Kβ activation (Garcia et al., 2010). The role of PI3Kβ in the synergy between gAd and G_i/G_z signalling pathways is not known, but may involve downstream activation of PI3K substrates, which include Akt and Rap1b. Akt is a serine-threonine kinase composed of a centrally located catalytic domain connected to a short C-terminal tail to a PH domain (Kim et al., 2004). Three isoforms of Akt are expressed in platelets, Akt1, Akt2 and Akt3 in which Akt1 is the most abundantly expressed (Cho et al., 2001, Woulfe et al., 2004). Genetic deletion of Akt1 isoform in mice have shown reduced platelet responses to thrombin and collagen, characterised with reduced secretion of ATP and increased tail-bleeding time (Chen et al., 2004). Loss of Akt2 resulted in impaired platelet responses to PAR4 agonist but not to collagen (Woulfe et al., 2004). These mice showed no change in tail-bleeding time and therefore suggested Akt1 is the major isoform involved in normal platelet function. The results of this

study suggest that gAd also stimulates Akt1 activation through the phosphorylation of serine^{473} residue.

It is established that G_i signalling downstream of $P2Y_{12}$ and α_{2A}-adrenoceptor activation leads to the activation of small GTPase Rap1 isoforms. Platelets highly express the Rap1b isoform, which can account for up to 0.1% of cellular protein (Torti and Lapetina, 1994). The activation of Rap1b has been implicated in the stabilisation of integrin $\alpha_{IIb}\beta_3$ activation and therefore can potentiate aggregation (Lova et al., 2003). The exact role of Rap1b in this process is unclear but may involve class IA PI3K isoforms. In fact, the activation of Rap1b has been shown to be PI3K dependent (Lova et al., 2002, Lova et al., 2003). More recent study has identified PI3Kβ as a key regulator of Rap1b activation downstream of GPVI activation (Larson et al., 2003, Jackson et al., 2005, Canobbio et al., 2009). Jackson and colleagues showed that the inhibition of PI3Kβ by TGX-221 can reduce Rap1b activation by 70% in G_i-stimulated platelets (Jackson et al., 2005). It is therefore possible that a similar pathway involving PI3Kβ downstream of gAd and G_i/G_z signalling can potentiate platelet aggregation.

The mechanism underlying PI3K induced granule secretion is not understood, but may involve the potentiation of TxA_2 generation. It has been shown that co-stimulation of G_i and G_z pathways in the absence of G_q signalling can lead to enhanced platelet aggregation and TxA_2 generation (Garcia et al., 2010). It is established that ADP-induced TxA_2 generation occurs through the regulation of extracellular signal-regulated kinase (ERK) activation (Garcia et al., 2007). A recent study has shown that PI3Kβ has a critical role in the ADP-induced TxA_2 generation through the regulation of ERK activation (Garcia et al., 2010). In fact, the study showed that co-stimulation of G_i/G_z by ADP and adrenaline enhanced TxA_2 generation via a pathway mediated by PI3Kβ (Garcia et al., 2010). We have previously shown that gAd and G_i/G_z signalling can synergise to enhance platelet dense-granule secretion independently of PKC. In conjunction to this, we have also shown that the release of TxA_2 is required for granule secretion in gAd-stimulated platelets. It is therefore possible that platelet dense-

granule secretion is potentiated via the increase in TxA_2 generation through a pathway involving ERK and PI3Kβ activation [Figure 4.31]. This mechanism may suggest two waves of ADP secretion, which may potentiate platelet responses to gAd. The first wave of ADP secretion is induced by direct activation of PLA_2 by primary gAd signalling which can drive ADP release. A second wave of ADP secretion is induced by G_i activation of PI3Kβ leading to increased TxA_2 generation and subsequent ADP release, which may potentiate aggregation. This hypothesis can easily be tested through the measurement of TxA_2 or ATP in the presence of the PI3K isoform inhibitors in PKC independent conditions. In conclusion, this study has established PI3Kβ as a major contributor to gAd-stimulated aggregation and secretion. Furthermore, we have identified a pathway involving PI3Kβ activation downstream of G_i/G_z signalling in the potentiation of platelet responses to gAd.

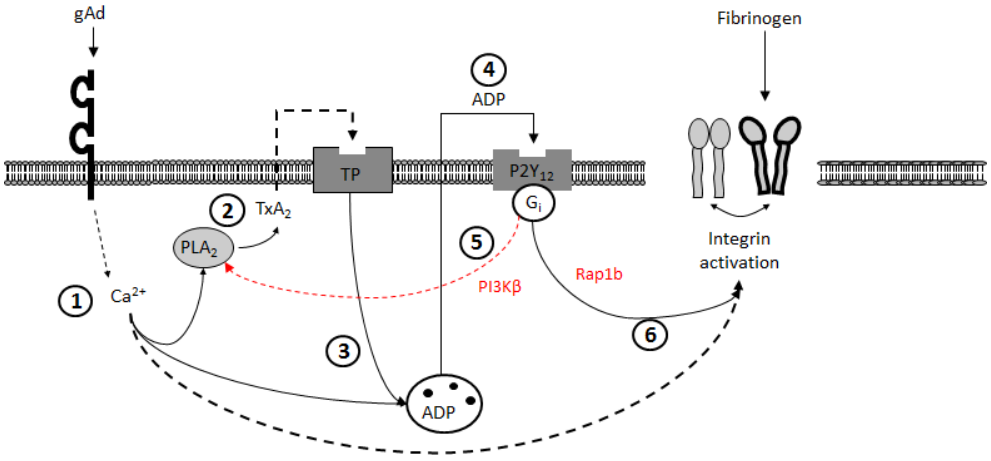

Figure 4.31: Proposed model for PKC independent secretion involving PI3Kβ. GAd signalling **(Step 1)** can synergise with G_i-induced activation of PI3Kβ to compensate for the loss of PKC signalling. TxA_2 formation **(Step 2)** may be up-regulated by a pathway involving PI3Kβ **(Step 5)**, which can increase ADP release from platelet-dense granules **(Step 3 and 4)**. Enhanced G_i signalling leads to stable and sustained integrin activation **(Step 6)**.

Chapter 5: Globular adiponectin: A novel collagen receptor glycoprotein VI agonist?

5.1 Introduction

In this study we have identified gAd as a novel platelet agonist that can stimulate platelet aggregation. However, the mechanism or receptor(s) involved in gAd-stimulated platelet activation is less clear. Most of our understanding of platelet activation by gAd stems from a study by Riba et al., (2008). This study used a genetic and non-genetic approach to suggest that gAd induced platelet activation through the collagen receptor GPVI-Fc chain complex. This was based on two major pieces of evidence, (i) platelet activation was associated with PLCγ2 activation through pathways involving Src-kinase isoforms, Syk and LAT in human platelets, and a lack of response in mice deficient in these signalling enzymes, and (ii) the activation of a NFAT linked signalling pathway downstream of transfected GPVI in Jurkat cells. Those findings suggested a new physiological GPVI agonist other than collagen and may represent a new group of proteins that can activate GPVI. However, that study failed to demonstrate a key role of GPVI in gAd-stimulated platelets. In this chapter we will focus on the role of collagen receptors, GPVI and integrin $\alpha_2\beta_1$ in gAd-stimulated platelet aggregation and adhesion by using specific receptor blocking antibodies.

5.2 The role of collagen glycoprotein VI receptor in globular adiponectin-stimulated aggregation

In this study we used GPVI blocking antibody, 10B12 (kind gift from Prof. Richard Farndale) and integrin $\alpha_2\beta_1$ blocking antibody, 6F1 (kind gift from Prof. Barry Coller) to assess the role of these receptors in gAd-stimulated aggregation. In the first instance, the antibodies were tested using standard aggregation experiments. The addition of 10B12 (20 μg/ml) did not cause inhibition of platelet aggregation. Pre-incubation of platelets with 10B12 (20 μg/ml) prior to the addition of gAd (20 μg/ml) did not affect aggregation stimulated by the adipokine [Figure 5.1A]. Interestingly, 10B12 also failed to influence collagen (1 μg/ml)-stimulated aggregation [Figure 5.1C]. In contrast to these data 10B12 abolished CRP-XL (5 μg/ml)-stimulated aggregation [Figure 5.1B].

The monoclonal antibody, 6F1 has been shown to inhibit platelet adhesion and aggregation induced by $\alpha_2\beta_1$ specific agonists (Verkleij et al., 1998, Jarvis et al., 2002). However, incubation of platelets with 6F1 (10 μg/ml) failed to influence aggregation by any of the agonists tested [Figure 5.1].

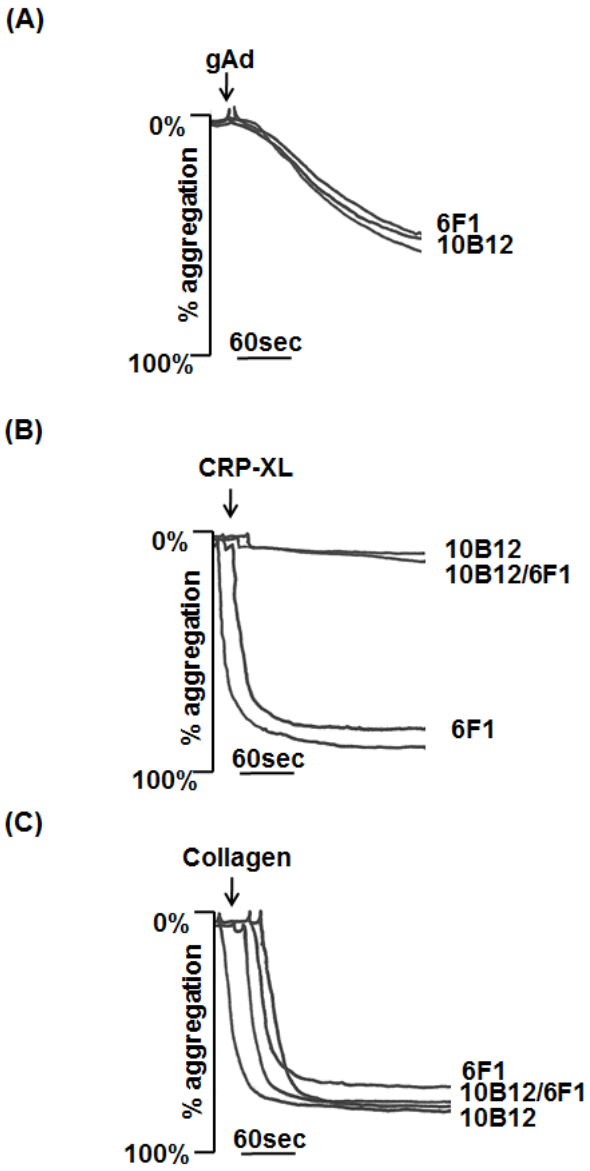

Figure 5.1: The role of glycoprotein VI in globular adiponectin-stimulated platelet aggregation. (**A**) Washed platelets ($2.5x10^8$ platelets/ml) were stimulated with gAd (20 µg/ml) in the absence and presence of 10B12 (20 µg/ml) and 6F1 (10 µg/ml) and the aggregation response was monitored for 4 minutes under constant stirring. (**B**) As in (A) except platelets were stimulated with CRP-XL (5 µg/ml) (**C**) As in (A) except platelets were stimulated with collagen (1 µg/ml). All representative aggregation traces are taken from three independent experiments performed with distinct blood donors.

5.3 Immobilised globular adiponectin supports platelet adhesion

The exposure of components of the extracellular matrix is an important event in the formation of a haemostatic plug. Initial experiments were designed to assess platelet adhesion to immobilised gAd under static conditions since plasma adiponectin has been found immobilised to collagen fibres (Okamoto et al., 2002). Here, gAd was immobilised on glass slides and washed platelets were allowed to adhere for 1 hour and stained with TRITC-conjugated phalloidin and viewed under x60 oil immersion lens in an inverted microscope.

We found washed platelets adhered to immobilised gAd in a concentration-dependent manner [Figure 5.2]. Immobilised gAd (20 μg/ml) supported platelet adhesion of 604±89 platelets/0.1mm^2 and optimal adhesion was observed with gAd (40 μg/ml) of 1087±146 platelets/0.1mm^2, although the platelets underwent a limited spreading response. Importantly, platelets adhered to immobilised gAd were shown to have undergone secretion in a concentration-dependent manner. It was found that adherent platelets showed extensive staining for P-selectin [Figure 5.2]. Taken together, these data suggest that immobilised gAd can support platelet adhesion, but does not induce a significant spreading response [Figure 5.2B].

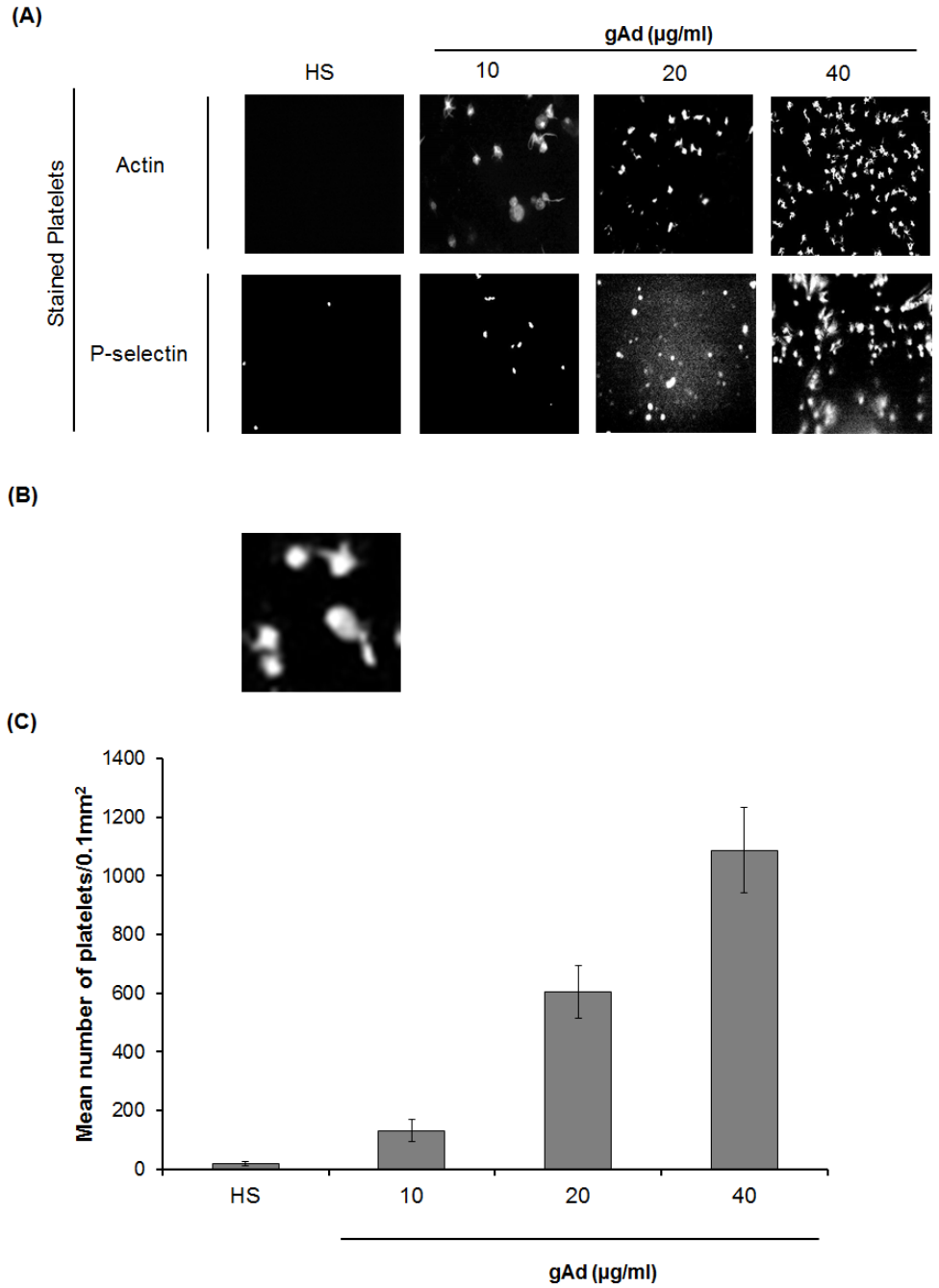

Figure 5.2: Immobilised globular adiponectin supports platelet adhesion. (**A**) Glass microscope slides were coated overnight with increasing concentrations of gAd and washed platelets ($5x10^7$ platelets/ml) were allowed to adhere for 1 hour at 37°C. Adherent platelets were stained with TRITC-conjugated phalloidin (top panel) or p-selectin (bottom panel) and viewed through x60 oil immersion lens. (**B**) Platelets show limited spreading on immobilised gAd. (**C**) Platelets from 8 random visual fields with a total area of $0.1mm^2$ were counted and expressed as mean number of platelets/$0.1mm^2$ ± SEM of three independent experiments performed with distinct blood donors.

5.3.1 Platelet adhesion induced by globular adiponectin is tyrosine kinase-dependent

Since gAd-stimulated platelet activation is tyrosine kinase-dependent, a pan Src-kinase inhibitor, PP1 was used to assess platelet adhesion to gAd. In these conditions, gAd (40 μg/ml) supported similar levels of platelet adhesion of 1290±210 platelets/0.1mm^2 found with previous experiment [Figure 5.3]. Incubation of platelets with PP1 significantly reduced platelet adhesion to 340±100 platelets/0.1mm^2 ($p<0.05$). Importantly, the inactive analogue of the Src-kinase inhibitor, PP3 (20 μM) did not affect platelet adhesion [Figure 5.3]. Furthermore, treatment of platelets with EGTA (1 mM) to prevent integrin dependent conditions did not ablate platelet adhesion [Figure 5.3]. Taken together, these data show that platelet adhesion to immobilised gAd occurs through a tyrosine kinase-dependent pathway that was independent of surface integrins.

We next examined platelet adhesion induced by other agonists that induce platelet activation by tyrosine kinase-dependent manner, such as CRP-XL and collagen. Consistent with previous published reports (Shadle and Barondes, 1982, Nakamura et al., 1998), collagen (10 μg/ml) supported platelet adhesion of 940±130 platelets/0.1mm^2 which was characterised by robust platelet spreading [Figure 5.4]. The addition of PP1 reduced platelet adhesion to 610±260 platelets/0.1mm^2 and to 663±223 platelets/0.1mm^2 with the addition of EGTA [Figure 5.4]. In comparison, CRP-XL (10 μg/ml) stimulated platelet adhesion of 1785±455 platelets/0.1mm^2 and was significantly reduced to 499±158 platelets/0.1mm^2 by PP1 ($p<0.05$) [Figure 5.5]. It is important to note that like gAd and CRP-XL induced adhesion was also unaffected by EGTA, whereas collagen was affected. These data suggest that GPVI activation alone can support platelet adhesion.

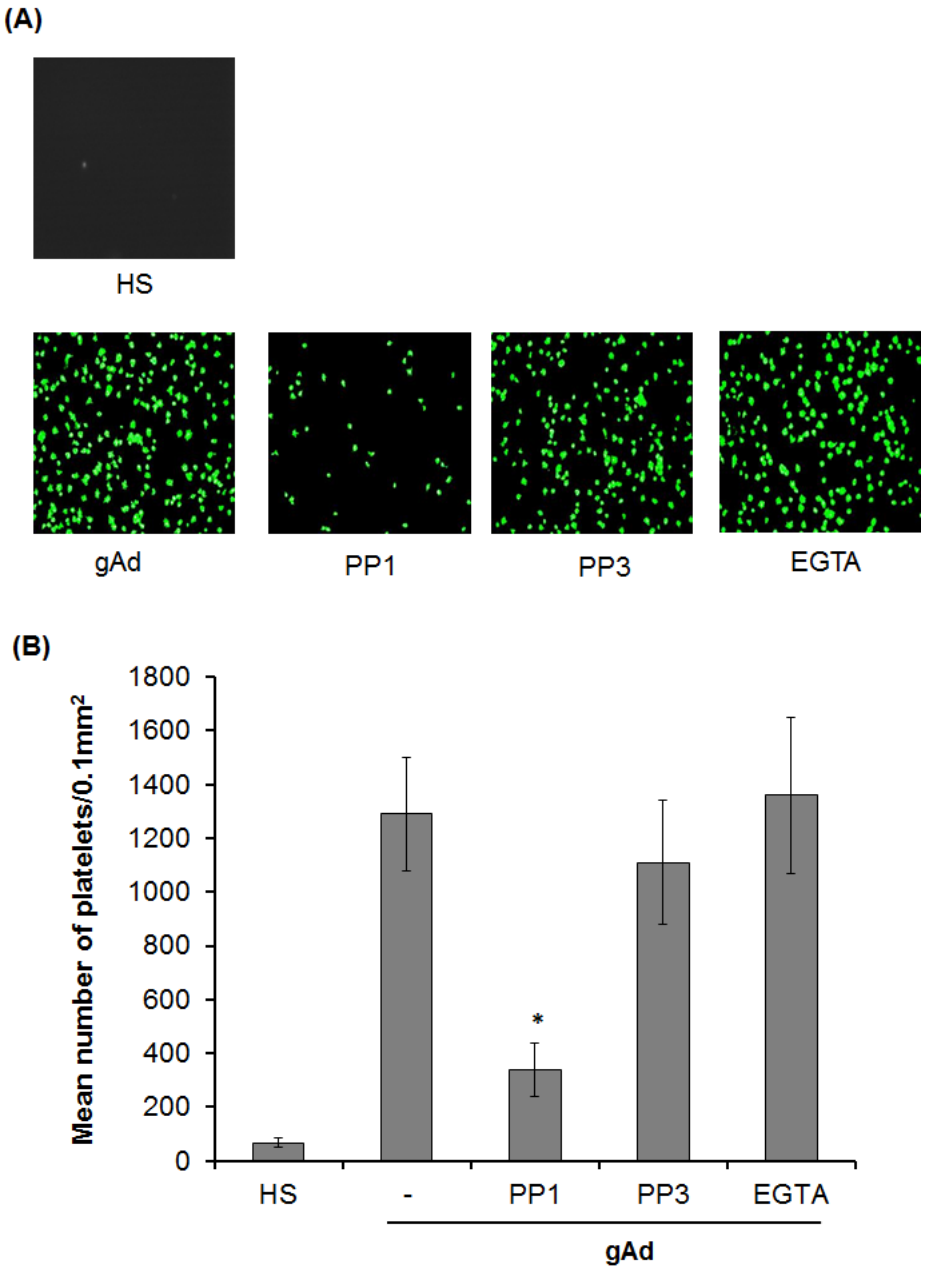

Figure 5.3: Platelet adhesion to immobilised globular adiponectin is tyrosine kinase-dependent. (**A**) Washed platelets ($5x10^7$ platelets/ml) were treated with PP1 (20 µM), PP3 (20 µM) or EGTA (1 mM) for 20 minutes at 37°C then adhered for 1 hour at 37°C on glass microscope slides coated with gAd (40 µg/ml) overnight. Adherent platelets were stained with TRITC-conjugated phalloidin and viewed through x60 oil immersion lens. (**B**) Platelets from 8 random visual fields with a total area of $0.1mm^2$ were counted and expressed as mean number of platelets/$0.1mm^2$ ± SEM of three independent experiments performed with distinct blood donors, where * indicates $p<0.05$ compared with gAd alone to the inhibitors.

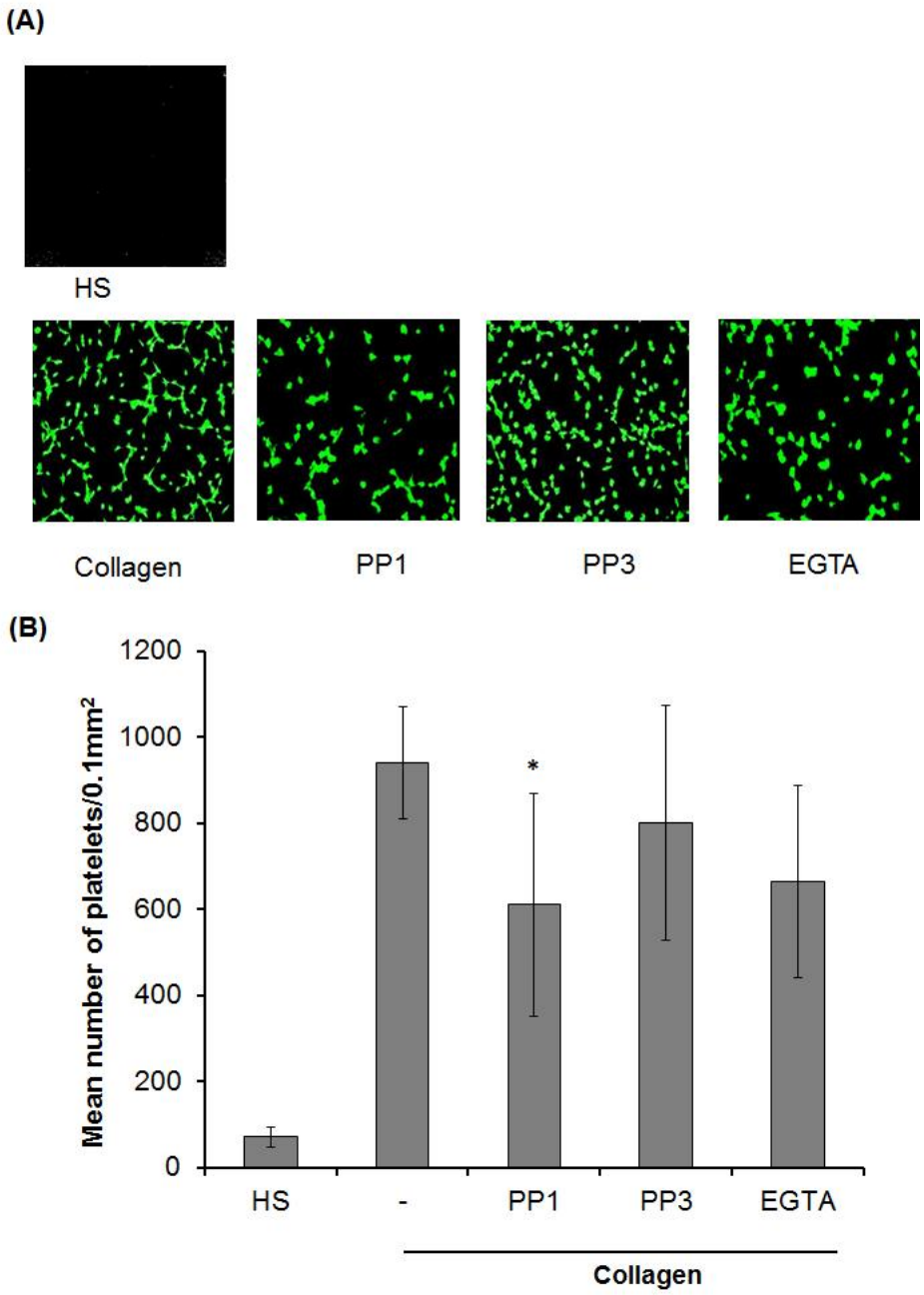

Figure 5.4: Platelet adhesion to immobilised collagen is tyrosine kinase-dependent. (**A**) Washed platelets ($5x10^7$ platelets/ml) were treated with PP1 (20 µM), PP3 (20 µM) or EGTA (1 mM) for 20 minutes at 37°C then adhered for 1 hour at 37°C on glass microscope slides coated with collagen (10 µg/ml) overnight. Adherent platelets were stained with TRITC-conjugated phalloidin and viewed through x60 oil immersion lens. (**B**) Platelets from 8 random visual fields with a total area of $0.1mm^2$ were counted and expressed as mean number of platelets/$0.1mm^2$ ± SEM of three independent experiments performed with distinct blood donors, where * indicates $p<0.05$ compared with collagen alone to the inhibitors.

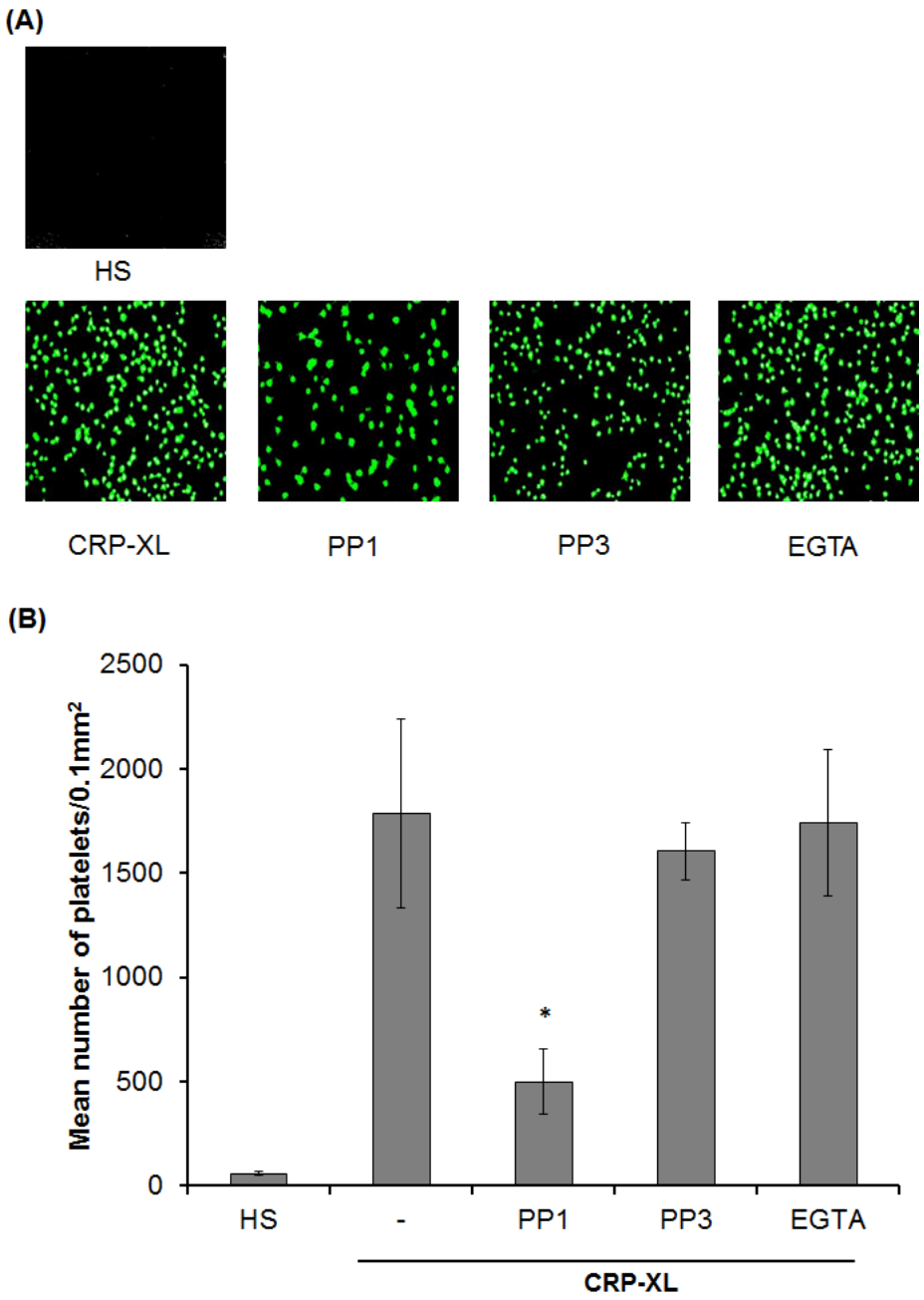

Figure 5.5: Platelet adhesion to immobilised CRP-XL is tyrosine kinase-dependent. (**A**) Washed platelets ($5x10^7$ platelets/ml) were treated with PP1 (20 µM), PP3 (20 µM) or EGTA (1 mM) for 20 minutes at 37°C then adhered for 1 hour at 37°C on glass microscope slides coated with CRP-XL (10 µg/ml) overnight. Adherent platelets were stained with TRITC-conjugated phalloidin and viewed through x60 oil immersion lens. (**B**) Platelets from 8 random visual fields with a total area of 0.1 mm^2 were counted and expressed as mean number of platelets/0.1mm^2 ± SEM of three independent experiments performed with distinct blood donors, where * indicates $p<0.05$ compared with CRP-XL alone to the inhibitors.

5.3.2 Glycoprotein VI mediates globular adiponectin-stimulated platelet adhesion

While our data did not confirm a role of GPVI in gAd-stimulated aggregation, we examined the role of this receptor in gAd-stimulated adhesion since the conformation of the protein can be potentially altered when immobilised. Washed platelets were incubated in the presence of 10B12 (20 μg/ml) or 6F1 (10 μg/ml) and were allowed to adhere for 1 hour on immobilised gAd (40 μg/ml). The presence of 10B12 (20 μg/ml) significantly reduced platelet adhesion from 560±110 to 170±20 platelets/0.1mm^2 ($p<0.05$) [Figure 5.6]. In contrast, the inhibition of integrin $\alpha_2\beta_1$ by 6F1 (10 μg/ml) did not influence platelet adhesion [Figure 5.6]. Importantly, platelet adhesion was reduced to 100±20 platelets/0.1mm^2 ($p<0.05$) in the presence of both receptor blocking antibodies [Figure 5.6]. These data may suggest a role of GPVI in platelet adhesion to immobilised gAd.

To further understand the ability of 10B12 to block GPVI mediated platelet adhesion, the above experiment was repeated with collagen and CRP-XL. Consistent with the ability of 10B12 to abolish CRP-XL-stimulated aggregation, platelet adhesion was abolished with the addition of 10B12 (20 μg/ml) from 2790±90 to 120±40 platelets/0.1mm^2 ($p<0.05$) [Figure 5.7]. In contrast, the GPVI blocking antibody was found to have a minor effect on collagen (10 μg/ml)-stimulated platelet adhesion [Figure 5.8]. However, the addition of 6F1 reduced platelet adhesion from 3400±330 to 1750±60 platelets/0.1mm^2 and similar levels were found when the two blocking antibodies were combined [Figure 5.8].

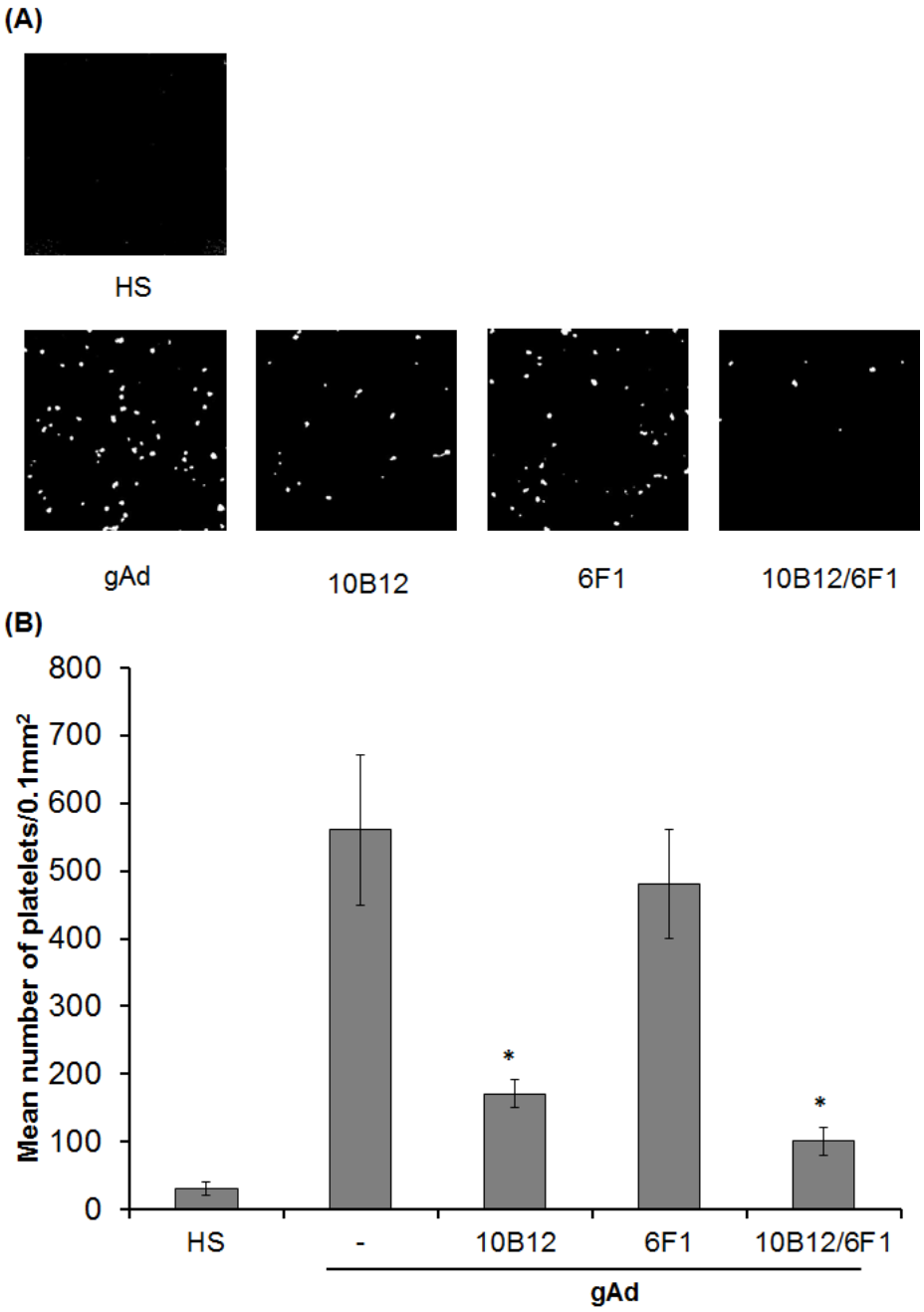

Figure 5.6: The role of glycoprotein VI in platelet adhesion to immobilised globular adiponectin. (**A**) Washed platelets ($5x10^7$ platelets/ml) were treated with 10B12 (20 µg/ml) or 6F1 (10 µg/ml) for 20 minutes at 37°C then adhered for 1 hour at 37°C on glass microscope slides coated with gAd (40 µg/ml) overnight. Adherent platelets were stained with TRITC-conjugated phalloidin and viewed through x60 oil immersion lens. (**B**) Platelets from 8 random visual fields with a total area of $0.1mm^2$ were counted and expressed as mean number of platelets/$0.1mm^2$ ± SEM of

three independent experiments performed with distinct blood donors, where * indicates $p<0.05$ compared with gAd alone to the inhibitors.

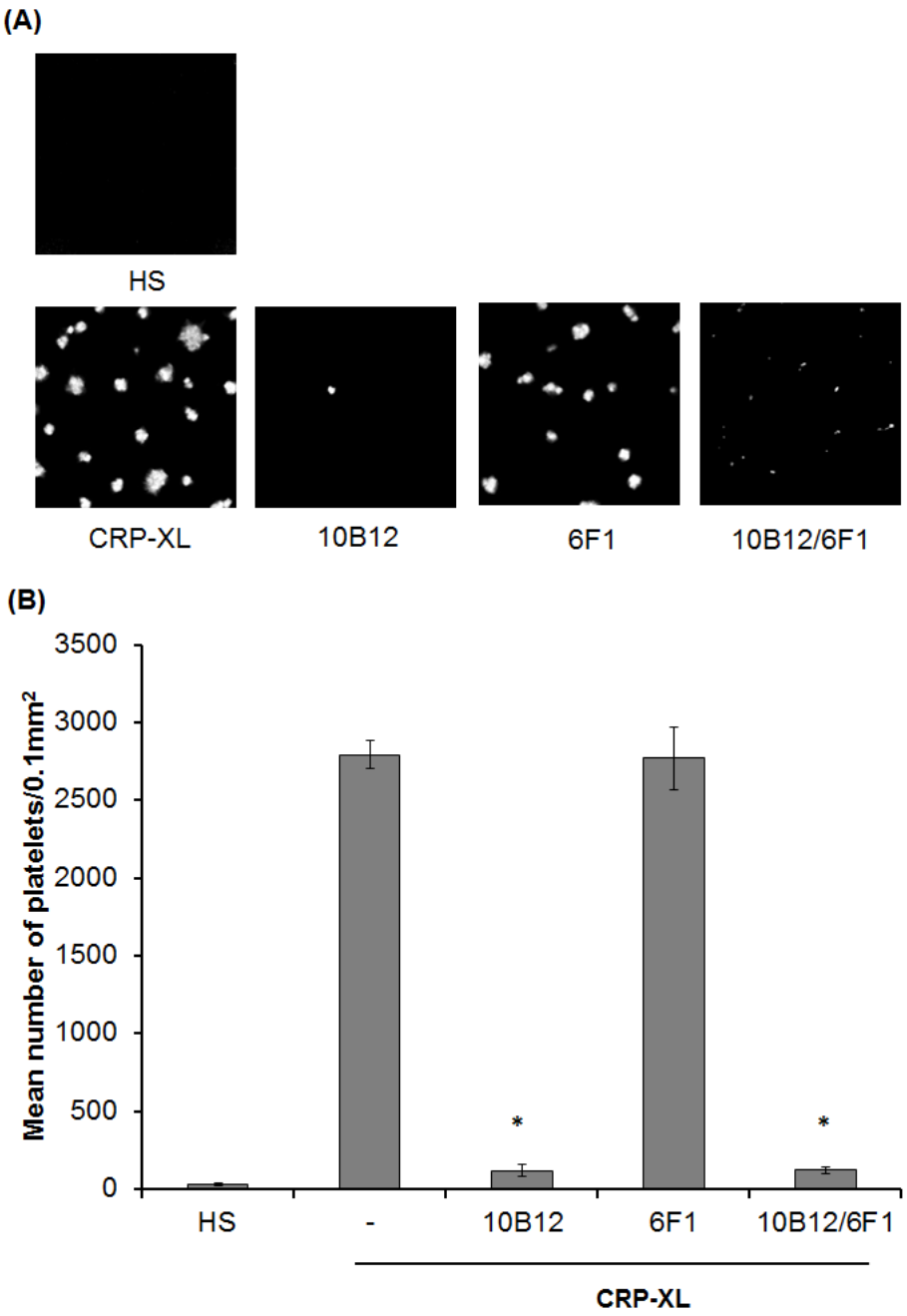

Figure 5.7: The role of glycoprotein VI in platelet adhesion to immobilised CRP-XL. (**A**) Washed platelets ($5x10^7$ platelets/ml) were treated with 10B12 (20 µg/ml) or 6F1 (10 µg/ml) for 20 minutes at 37°C then adhered for 1 hour at 37°C on glass microscope slides coated with CRP-XL (10 µg/ml) overnight. Adherent platelets were stained with TRITC-conjugated phalloidin and viewed through x60 oil immersion lens. (**B**) Platelets from 8 random visual fields with a total area of $0.1mm^2$ were counted and expressed as mean number of platelets/$0.1mm^2$ ± SEM of three

independent experiments performed with distinct blood donors, where * indicates $p<0.05$ compared with CRP-XL alone to the inhibitors.

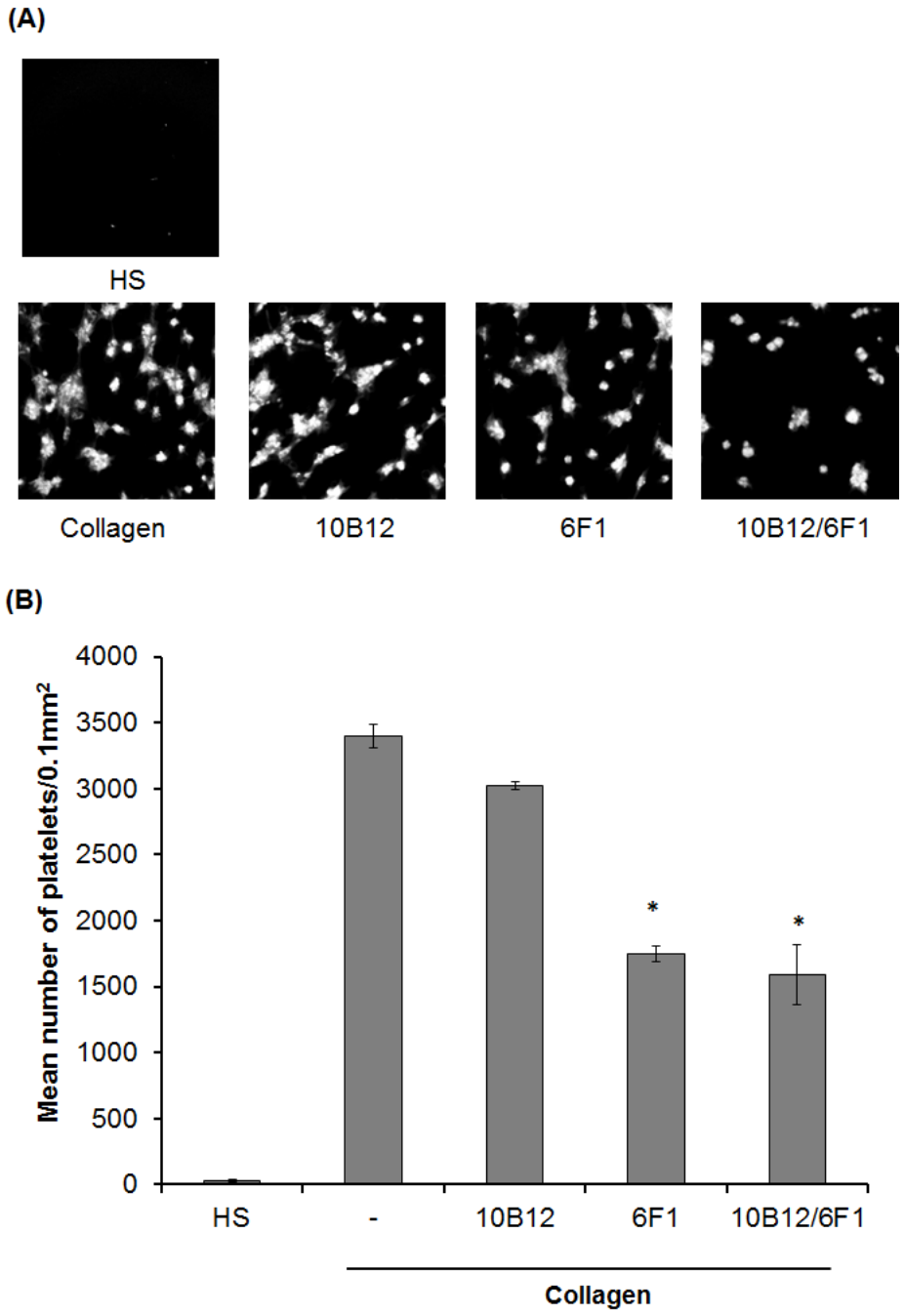

Figure 5.8: The role of glycoprotein VI in platelet adhesion to immobilised collagen. (**A**) Washed platelets ($5x10^7$ platelets/ml) were treated with 10B12 (20 µg/ml) or 6F1 (10 µg/ml) for 20 minutes at 37°C then adhered for 1 hour at 37°C on glass microscope slides coated with collagen (10 µg/ml) overnight. Adherent platelets were stained with TRITC-conjugated phalloidin and viewed through x60 oil immersion lens. (**B**) Platelets from 8 random visual fields with a total area of $0.1mm^2$ were counted and expressed as mean number of platelets/$0.1mm^2$ ± SEM of three

independent experiments performed with distinct blood donors, where * indicates $p<0.05$ compared with collagen alone to the inhibitors.

5.3.3 Immobilised globular adiponectin supports platelet adhesion under flow conditions

Platelet adhesion is an important event that supports the formation of the haemostatic plug, therefore preliminary experiments were performed to examine the ability of gAd to support platelet adhesion under flow conditions. Whole blood was flowed over immobilised gAd (40 μg/ml) at two different shear rates, $100s^{-1}$ to mimic venous shear rate and at $1000s^{-1}$ to mimic shear rate found in the arteries.

At the lower flow rate of $100s^{-1}$, gAd supported platelet adhesion and thrombus formation. This was characterised by 4.78% surface coverage and thrombus mean height of 0.15 μm [Figure 5.9]. For comparison, collagen (10 μg/ml) stimulated a more robust platelet adhesion and thrombus formation, which corresponded to 12.75% surface coverage and 0.26 μm mean thrombus height [Figure 5.9]. The experiments were then repeated at a higher flow rate of $1000s^{-1}$ to mimic arterial shear. Under these conditions we found platelet adhesion and thrombus formation to immobilised gAd was less stable [Figure 5.9]. Interestingly surface coverage was maintained to a similar level of 4.1%, but there was a reduction of mean thrombus height to 0.06 μm when compared to the lower flow rate of $100s^{-1}$ [Figure 5.9]. Taken together, this preliminary data suggest that immobilised gAd can support platelet adhesion and thrombus formation, but is most effective at lower shear rates.

Adiponectin is found immobilised to components of the extracellular matrix (Okamoto et al., 2002). Thus it seemed reasonable to examine if gAd could influence platelet adhesion to other known thrombogenic matrix proteins. To test this, gAd (40 μg/ml) was immobilised on a combination of vWF type III (10 μg/ml) and the integrin $\alpha_2\beta_1$ specific agonist, glycine-phenylalanine-hydroxyproline-glycine-glutamine-arginine (GFOGER) (10 μg/ml) (Pugh et al., 2010). Under conditions that mimic arterial shear rate ($1000s^{-1}$), gAd alone supported weak platelet adhesion characterised by 4.78% surface coverage and 0.06 μm mean thrombus height [Figure 5.9]. In

contrast, immobilised vWF-III and GFOGER supported robust platelet adhesion and thrombus formation at this high shear rate, which led to 10.12% increase in surface coverage and 0.2 µm mean thrombus height. Interestingly, the combination of immobilised gAd with vWF-III and GFOGER potentiated thrombus formation [Figure 5.9]. This was associated with increased platelet adhesion characterised by 19.29% surface area coverage and a mean thrombus height of 0.36 µm [Figure 5.9]. Taken together, these data suggest that immobilised gAd on extracellular matrix proteins can support enhanced platelet adhesion and thrombus formation under flow conditions. These data suggest that gAd-stimulated platelet activation can synergise with other signalling pathways downstream of GPIb-V-IX and integrin $\alpha_2\beta_1$ to enhance thrombus formation. These results may represent a new pathological role for gAd in thrombosis.

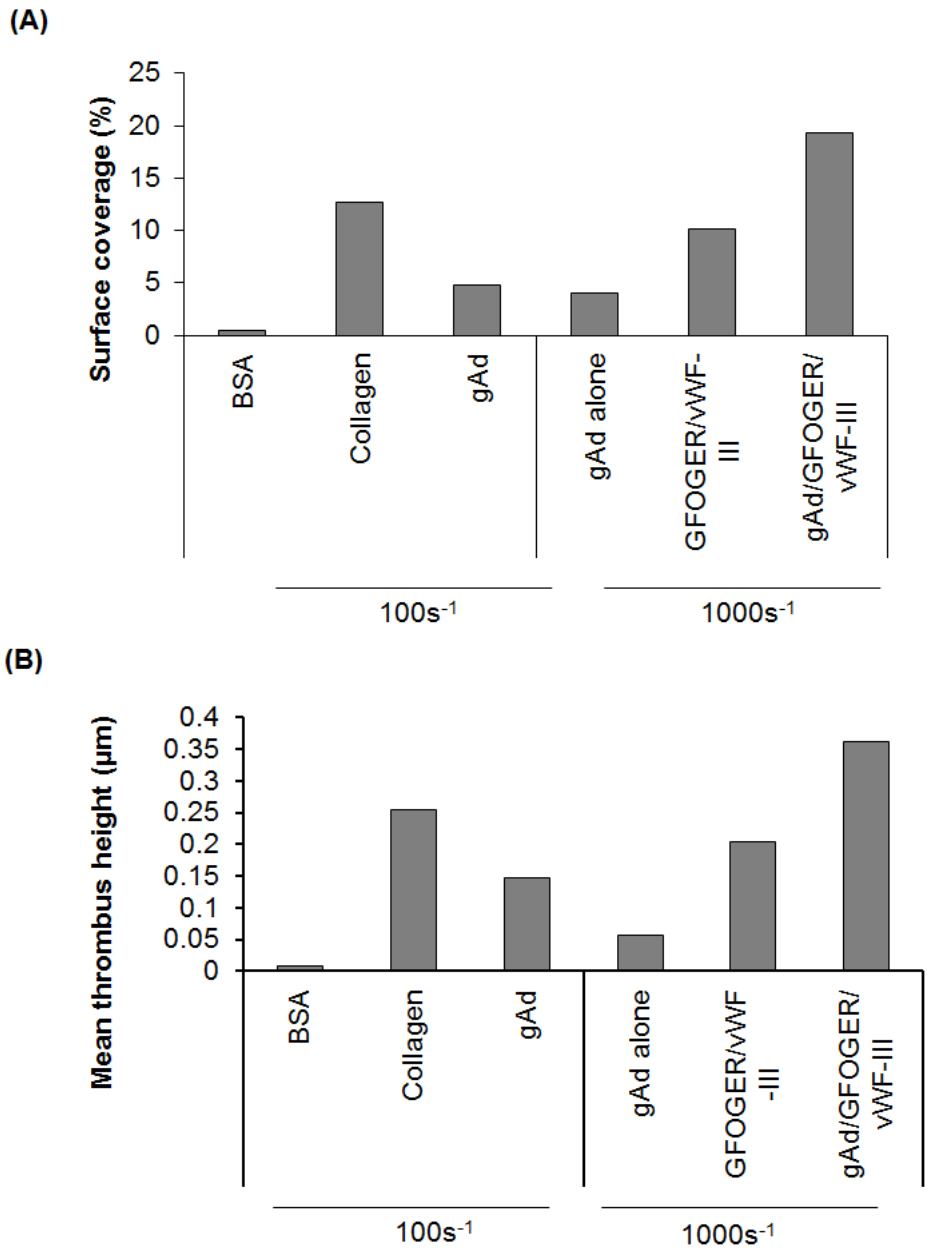

Figure 5.9: Immobilised globular adiponectin enhances platelet adhesion and thrombus formation by sub-endothelial proteins. (**A**) $DiOC_6$ (1 µM) labelled whole blood was perfused over immobilised gAd (40 µg/ml) alone or co-immobilised with vWF-III (10 µg/ml) and GFOGER (10 µg/ml) for 5 minutes at the indicated shear rates and viewed by a fluorescent confocal microscope. Image J software (NIH) was used to measure percentage surface coverage. (**B**) As in (A) except data was expressed as mean thrombus height.

5.4 Discussion

The activation of platelets at the sites of vascular damage is an essential step in haemostasis. These events are initiated by interaction of platelets with components of the extracellular matrix such as collagen with its receptors, GPVI and integrin $\alpha_2\beta_1$ (Nieswandt and Watson, 2003, Farndale et al., 2004, Watson et al., 2005). In this study we have identified gAd as a novel platelet agonist that can activate platelets in a tyrosine kinase-dependent manner. An important study by Riba et al., suggested that the GPVI-Fc receptor chain complex may be involved in gAd-stimulated platelet activation (Riba et al., 2008). In this chapter preliminary experiments were performed to examine the role of the collagen receptors, GPVI and $\alpha_2\beta_1$ in platelet responses induced by gAd stimulation.

Selective receptor blocking antibodies were used to establish the role of GPVI and integrin $\alpha_2\beta_1$ in gAd-stimulated aggregation. The GPVI blocking antibody, 10B12 was obtained with screening of phage display libraries against the Ig-like domain of GPVI (Smethurst et al., 2004). The association between the collagen GPO repeat motif and the GPVI is disrupted upon the addition of 10B12. In contrast, the integrin $\alpha_2\beta_1$ blocking antibody, 6F1 disrupts the association between collagen and the integrin I domain (Coller et al., 1989, Nieswandt and Watson, 2003). We found that 10B12 abolished CRP-XL-stimulated aggregation, but did not affect gAd or collagen-stimulated aggregation. However, 10B12 did significantly reduce platelet adhesion to immobilised gAd. Platelet GPVI activation by collagen involves the interaction of collagen to multiple binding sites found within GPVI receptor (Nieswandt and Watson, 2003, Smethurst et al., 2004, Smethurst et al., 2007). The interaction between 10B12 and GPVI has been mapped to the apical region of the GPVI receptor and was able to abolish CRP-XL-stimulated aggregation. Since collagen-stimulated aggregation was not affected by 10B12 it may suggest that different binding sites are involved in GPVI activation. For example, 10B12 has been shown to be most effective against platelet stimulation with CRP-XL. It is thought that the 10B12 epitope overlaps with the CRP-XL binding site (Smethurst et al., 2004). It is

also important to consider that the possible interaction between gAd and GPVI may be GPO independent and may represent a new GPVI binding site which allows platelet activation by gAd. Therefore, the use of GPVI blocking antibody was unable to confirm the role of GPVI in gAd-stimulated aggregation. The use of an alternative GPVI blocking antibody which can disrupt the activation and clustering of GPVI could have been used. However, with the emergence of GPVI knock-out mice, these animals would have provided the experimental tools needed to investigate the role of GPVI in gAd-stimulated activation. However, platelet activation by gAd was found to be independent of integrin $\alpha_2\beta_1$ activation. In contrast, platelet adhesion to collagen is both GPVI and integrin dependent (Nieswandt et al., 2001, Nieswandt and Watson, 2003). Further work is needed to determine the exact role of GPVI in gAd-induced platelet activation.

The initial step in the haemostatic process is the platelet adhesion with exposed elements of extracellular matrix proteins such as collagen or vWF at the site of vascular injury. In the latter part of this chapter, we examined the ability of platelets to adhere to immobilised gAd under static and flow conditions. We found that platelets adhered to immobilised gAd in a concentration-dependent manner. Furthermore, we found platelet adhesion to immobilised gAd was tyrosine kinase-dependent. In contrast, platelet adhesion to immobilised gAd was integrin-independent. Interestingly, the addition of 10B12 was able to ablate adhesion and therefore suggests that GPVI is involved in platelet adhesion to gAd. However, the addition of 10B12 was not able to inhibit gAd-stimulated platelet aggregation in suspension. The immobilisation of gAd to a glass surface may slightly alter the protein tertiary structure to expose new binding sites for GPVI interaction which can be blocked by 10B12. It is widely known that both fibrinogen and vWF undergo a similar conformation change that supports platelet adhesion (Ugarova et al., 1993, Siediecki et al., 1996). However, further work is needed to understand possible gAd interaction with GPVI in platelet adhesion.

It was important to understand if gAd can support platelet adhesion and thrombus formation under flow conditions. We found immobilised gAd was able to support stable platelet adhesion at low shear rate ($100s^{-1}$). In contrast, platelet adhesion was significantly reduced at high shear rate ($1000s^{-1}$). This data is consistent with other GPVI specific agonist such as CRP-XL that can only support weak platelet adhesion under flow at high shear (Nieswandt and Watson, 2003). Importantly, immobilisation of vWF-III, integrin $\alpha_2\beta_1$ agonist GFOGER and gAd was able to potentiate thrombus formation. These data suggest that platelet adhesion to gAd at high shear rate is weak and the stimulation of additional receptors is required to reinforce platelet adhesion and thrombus formation.

These findings have established gAd as a novel platelet agonist that can support both platelet aggregation and adhesion. Plasma adiponectin has been found immobilised to components of extracellular matrix and therefore may support platelet adhesion with the globular domain. However, since full-length form of adiponectin did not stimulate platelet aggregation, it is unlikely if this is the case. It is more likely that the generation of gAd is promoted at sites of vascular inflammation such as atherosclerosis and can potentially become immobilised to the exposed components of the extracellular matrix. In this micro-environment, immobilised gAd in conjunction with collagen or vWF can cause unwanted platelet adhesion or thrombosis. However, more work is needed to understand these pathological roles of gAd in the vascular system.

Chapter 6: General discussion

Adiponectin is the major adipokine produced by the adipose tissue and has complex and varied biological functions. Its primary role is as an insulin-sensitising hormone and therefore plays a role in carbohydrate and lipid metabolism. However, as previously stated anti-diabetic, anti-inflammatory and vascular protective actions have also been described (Okamoto et al., 2002, Ouchi and Walsh, 2007, Zhu et al., 2008). Several groups have sought to examine the role of adiponectin on platelet function and have proposed that adiponectin has anti-thrombotic properties (Kato et al., 2006, Restituto et al., 2010). An important study by Kato and colleagues proposed that adiponectin may act as an endogenous anti-thrombotic factor, thereby limiting thrombus formation (Kato et al., 2006). However, it was unclear if this anti-thrombotic property was due to the direct effect of adiponectin on platelet function or by increasing vascular NO production and thereby increasing NO bioavailability. However, this study while important, may have been over simplistic in its approach to the effects of adiponectin on platelet function, since it failed to take into account the multiple forms of adiponectin in circulation. In this study we addressed this latter issue and aimed to understand the direct effect of gAd on platelet function.

The result of this investigation has established that only the globular form of adiponectin can stimulate platelet aggregation and adhesion, while the full-length form may play an inhibitory role (Restituto et al., 2010). This is surprising since fAd contains a collagen-like domain which can be recognised by the collagen receptor GPVI. It is not clear why the globular domain of fAd did not stimulate aggregation, although it is possible that binding of this collagen-like domain of fAd may create steric hindrance to prevent GPVI from being activated. Since these initial experiments established gAd as a novel platelet agonist, it was important to understand its mechanism of action. We found that gAd activated platelets in a tyrosine kinase-dependent manner. This was accompanied with the activation of Syk, PLCγ2, LAT and Src family kinase signalling pathways (Riba

et al., 2008). These data strongly suggest that gAd stimulates platelet activation through a similar pathway to that of collagen. It is established that the platelet activation by collagen involves two receptors, GPVI and integrin $\alpha_2\beta_1$. The activation of platelets by GPVI involves the formation of a tyrosine kinase-dependent signalosome that leads to the mobilisation of intracellular Ca^{2+} and integrin $\alpha_{IIb}\beta_3$ activation. The same study by Riba et al., (2008) proposed that gAd-stimulated platelet activation through the collagen receptor GPVI. In this study we were unable to confirm the role of GPVI in gAd-stimulated aggregation, since the best available GPVI blocking antibody failed to influence aggregation. However, GPVI activation was implicated in platelet adhesion to immobilised gAd. Further work is needed to determine the exact role of gAd in GPVI activation. It is important that we understand the interaction between gAd and GPVI which may represent a novel mechanism for GPVI activation that is independent of GPO motif.

Recombinant forms of adiponectin were used in this investigation and it was important to consider the recombinant nature of the protein. Importantly, no differences were found between recombinant or HEK-derived gAd in stimulating platelet aggregation. However, during the study we found variation between different batches of gAd with regards to their ability to stimulate platelet aggregation. After much troubleshooting, it became apparent that the addition of BSA was vital to its stability. This is consistent with the physiological situation since plasma adiponectin is found to circulate associated with albumin or other serum proteins (Wang et al., 2006, Hada et al., 2007). It is possible that this association may enhance its stability and allow greater access to sites of vascular damage or inflammation. The precise origin of gAd is unclear but may involve the cleavage of fAd by leukocyte elastase to produce the globular fragment (Waki et al., 2005). These findings can suggest the generation of gAd at sites of inflammation (Okamoto et al., 2000). Interestingly, gAd in contrast to fAd can have pro-inflammatory properties since it can increase expression of pro-inflammatory adhesion molecules, increase angiotensin-

II induced proliferation of cardiac fibroblasts (Hattori et al., 2006, Hattori et al., 2007) and stimulate THP-1 production by monocytes (Neumeier et al., 2006). Thus emerging evidence suggests that gAd may possess pro-inflammatory properties in addition to its insulin-sensitising effects (Hattori et al., 2006, Hattori et al., 2007). Consistent with these pathological roles, we have described gAd as a novel platelet agonist that can potentially increase unwanted platelet aggregation and thrombus formation in inflammatory conditions such as atherothrombosis. We have found gAd can support platelet adhesion and enhance thrombus formation through the interaction of collagen and vWF. Furthermore, platelet aggregation induced by gAd can be potentiated by ADP and adrenaline.

The release of platelet derived ADP is a crucial event that enables the additional recruitment of platelets and stabilisation of the growing thrombi (Kunapuli et al., 2003, Kim and Kunapuli, 2011). The release of ADP can also work in conjunction with less established agonists to potentiate aggregation. It is widely accepted that GPVI-stimulated aggregation can be potentiated with other agonists such as ADP (Atkinson et al., 2003). Consistent with these reports, ADP-dependent potentiation of platelet responses to gAd was found to involve the G_i-coupled $P2Y_{12}$ receptor. The exact mechanism involved in this G_i-dependent potentiation of platelet responses is not clear but may involve pathways linked to PI3K and PKC signalling. Therefore, it was important to understand the role of PI3K and PKC in gAd-stimulated aggregation. Consistent with our current understanding about the role of PKC on platelet aggregation, we found that gAd could stimulate aggregation through pathways dependent and independent of PKC activation. In contrast, gAd-stimulated secretion was found to be fully dependent on PKC signalling. Importantly, the aggregation response and dense-granule secretion under conditions of PKC inhibition could be partially restored by ADP acting through the $P2Y_{12}$ coupled G_i receptor and by adrenaline acting through the G_z-linked α_{2A}-adrenoceptor. These data have uncovered a relatively unknown pathway involved in PKC-independent secretion of platelet dense-granules. Further examination of

this pathway was found to require PI3Kβ activation and to a lesser extent PI3Kα isoform. These results suggest that a compensatory pathway may exist downstream of G_i/G_z-coupled receptors that can compensate for the loss of PKC signalling and can potentially synergise with other pathways to promote platelet activation and thrombus formation.

The activation of PI3K isoforms have been implicated in many platelet responses that include platelet shape change and aggregation (Rittenhouse, 1996, Pasquet et al., 1999). This study has identified a novel role for PI3Kβ isoform in ADP-dependent potentiation of platelet responses to gAd. In the last few years, the role of PI3Kβ in platelet function is becoming much clearer. The activation of PI3Kβ has been implicated in platelet integrin $\alpha_{IIb}\beta_3$ activation and adhesion (Jackson et al., 2005, Canobbio et al., 2009, Jackson and Schoenwaelder, 2010). This study has further contributed to our understanding to the role of PI3Kβ and may suggest a new possible target for anti-thrombotic agents. *In vivo* arterial thrombosis models have demonstrated that PI3Kβ inhibition can prevent occlusive thrombi from forming and had no effect on tail bleeding time (Jackson et al., 2005). It is possible that the inhibition of PI3Kβ may prevent multiple pathways involved in platelet activation and thrombus formation which may include the potentiation of platelet aggregation by ADP. Further work is needed to be done to understand the physiological significance of PI3Kβ activation in ADP potentiation of platelet aggregation and thrombus formation.

6.1 Conclusion

In conclusion, this study has identified gAd as a novel platelet agonist that can support platelet adhesion. Furthermore, we confirmed and extended our understanding of how gAd stimulates platelet aggregation. These findings question the proposed role of adiponectin as an anti-thrombotic agent (Kato et al., 2006). Our evidence taken with that of Kato and colleagues may suggest a dual role of adiponectin on platelet function. The native form may inhibit platelet aggregation and in contrast, the globular form can stimulate platelet aggregation. Thus, it is the relative balance of the different forms in circulation that may determine whether a pro or anti-thrombotic environment exists. Furthermore, it is important to determine if circulating levels of gAd are elevated in certain types of disease, particularly in early stages of atherosclerosis. It is also important to consider the indirect vascular effects of adiponectin on platelet function. Adiponectin is a potent stimulator of NO synthesis which can inhibit platelet activation. However, in inflammatory conditions like those found in atherosclerosis, the indirect effect of adiponectin on platelet function is limited. Arterial thrombosis is often determined by the local environment at the sites of vascular rupture. In this local environment the accumulation of adiponectin (Okamoto et al., 2002) and the potential generation of gAd may promote unwanted platelet aggregation and thrombus formation. Here the potential of fAd to stimulate increase NO synthesis would be of little benefit and the ability of gAd to synergise with ADP could be critical in promoting platelet aggregation.

6.2 Future work

In this study we have solely investigated the role of gAd on platelet function. However, due to time constraints not all aspects of gAd-induced platelet activation were investigated. Based on the experimental data several further aspects of study are warranted, including;

- Clarification of the receptors involved in gAd-stimulated platelet activation. This study was unable to confirm the role of GPVI in gAd-stimulated platelet activation due to the tools at our disposal. The role of GPVI in gAd-stimulated activation can be confirmed with the availability of GPVI null mice. These experiments would have allowed us to identify a new novel GPVI agonist. Furthermore, the possible interaction of gAd with GPVI would need to be closely examined.

- The use of pharmacological inhibitors is a powerful tool in understanding signalling pathways but some inhibitors can be non-specific. PKC and PI3K isoform specific knock-out mice would have allowed us to better understand the role of PI3Kβ in ADP-dependent potentiation of platelet responses.

- This study has proposed that platelet aggregation can be potentiated by ADP. It would be interesting to look at gAd ability to potentiate thrombus formation by an *in vivo* thrombosis model. This data would have proposed a new pathological role for gAd in the vascular system.

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Abbreviations

ACD	Acid citrate dextrose
Acrp30	Adipose complement related protein 30 kDa
ADP	Adensosine diphosphate
AMPK	AMP-activated protein kinase
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BAPTA-AM	1,2-bis (o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COX-1	Cyclo-oxygenase-1
CRP-XL	Cross linked-collagen related peptide
DAG	Diacylglycerol
$DiOC_6$	3,3' Dihexyloxacarbocyanine iodine
DTS	Dense tubular system
ECL	Enhanced chemiluminescence
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase

fAd	Full-length adiponectin
FcRγ-chain	Fc receptor gamma-chain
gAd	Globular adiponectin
Gads	Grb2 related protein downstream of Shc
GBP-28	Gelatine binding protein 28 kDa
GDP	Guanosine diphosphate
GFOGER	Glycine-phenylalanine-hydroxyproline-glycine-glutamine-arginine
GPO	Glycine-proline-hydroxyproline
GPVI	Glycoprotein VI
Grb2	Growth factor receptor bound protein 2
GTP	Guanosine triphosphate
HMW	High molecular weight isoform
HRP	Horseradish peroxidase
HS	Human serum
ICAM-1	Intracellular adhesion molecule
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
Indo	Indomethacin
IP_3	Inositol (1,4,5)-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
LAT	Linker for activation of T-cells
mAb	Monoclonal antibody

MAPK	p38 mitogen-activated protein kinase
MLC	Myosin light chain
NO	Nitric oxide
OCS	Open canalicular system
PAI-1	Plasminogen activator inhibitor type 1
PAR	Protease Activated Receptor
PBS	Phosphate buffered saline
PDK	Phosphoinositide-dependent kinase
PGI_2	Prostacyclin
PH	Pleckstrin homology
PI3K	Phophoinositol 3 kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone
PPAR	Peroxisome proliferator activated receptors
PRP	Platelet rich plasma
PVDF	Polyvinylidene difluoride
RBP4	Retinol binding protein 4

RGD	Arginine-glycine-serine
RGDS	Arginine-glycine-aspartic acid-serine
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
SH	Src homology
SLP76	SH2 domain containing leukocyte proteins of 76 kDa
SNARE	Soluble NEM-sensitive attachment protein receptors
Syk	Spleen tyrosine kinase
TBS-T	Tris buffered saline-tween
TEMED	N,N,N',N'-Tetramethyl ethylenediamine
TNF-α	Tumour necrosis factor-alpha
TxA_2	Thromboxane A_2
VCAM-1	Vascular cellular adhesion molecule-1
vWF	von Willebrand factor
Wort	Wortmannin